ORGAN-SPECIFIC EXPRESSION AND STRUCTURE OF RICE FERREDOXIN-NADP[+] OXIDOREDUCTASE GENES(Dissertation_全文)

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CONTENTS

Abbreviations

ATP	adenosine 5'-triphosphate
bp	base pair
CAT	chloramphenicol acetyltransferase
Cpn60	chaperon 60
CTAB	cetyl trimethyl ammonium bromide
CTP	cytosine 5'-triphosphate
DEPC	diethylpyrocarbonate
DIG	digoxigenin
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTSSP	3,3'-dithiobis(sulfosuccinimidyl)propionate
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDC	*N*-ethyl-3-[3-(dimethylamino)propyl]carbodiimide
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoreic mobility shift assay
Fd	ferredoxin
Fd_{ox}	oxidized ferredoxin
Fd_{red}	reduced ferredoxin
FNR	ferredoxin-$NADP^+$ oxidoreductase
GOGAT	glutamate synthase
GS	glutamine synthetase
GTP	guanosine 5'-triphosphate
GUS	β-glucuronidase
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
h	hour
Hsp70	70-kDa heat-shock proteins
IPTG	isopropyl-1-thio-β-D-galactoside
kb	kilobase
kDa	kilo dalton
2-ME	2-mercaptoethanol

min	minute
MOPS	2-(*N*-Morpholino)propanesulfonic acid
mRNA	messenger ribonucleic acid
M_r	molecular mass
N. crassa	*Neurospora crassa*
NiR	nitrite reductase
NR	nitrate reductase
NTP	nucleotide 5'-triphosphate
OPPP	oxidative pentose phosphate pathway
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
pBS	pBluescript® II
PCR	polymerase chain reaction
PGR	plant gene registers
pI	isoelectric point
PIPES	piperazine-*N*,*N*'-bis(2-ethansulfonic acid)
PMSF	phenylmethylsulfonyl fluoride
PS I	photosystem I
PS II	photosystem II
PVDF	polyvinylidene difluoride
PVP	polyvinyl pyroridone
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
SSC	sodium chloride/sodium citrate buffer
SDS	sodium dodecyl sulfate
TAE	Tris/acetic acid/EDTA buffer
TBE	Tris/boric acid/EDTA buffer
TE	Tris/EDTA buffer
Tris	tris(hydroxymethyl)aminomethane

CHAPTER I

INTRODUCTION

Function of ferredoxin-NADP$^+$ oxidoreductase in the liner photosynthetic electron transport system

In the oxygenic photosynthesis, the photosynthetic reaction involves the electron flow from H_2O to NADP$^+$ and all of the electron carriers exist in chloroplasts of higher plants. Most electrons generated in the photosynthesis are transported to Fd_{ox}. Fd is a M_r of 11 kDa protein, containing [2Fe-2S] cluster as a reaction center and interacts with a number of Fd-dependent enzymes such as FNR (EC 1.18.1.2), NiR (EC 1.7.7.1), GOGAT (EC 1.4.7.1), sulfite reductase (EC 1.8.7.1), Fd-thioredoxin reductase (EC 1.18.1.–) and stearoyl ACP desaturase (EC 1.14.99.6) as the central molecule for distributing electrons.

FNR is a monomeric flavoenzyme with M_r of 35 kDa which contains a single, noncovalently bound FAD as a prosthetic group (Sheriff et al. 1980). FNR catalyzes the final step of the liner photosynthetic electron transport system by mediating primarily the electron transfer from Fd_{red} to NADP$^+$ with formation of NADPH necessary for biosynthetic pathways. For the production of NADPH, excited electrons from PS I reduce the one electron carrying protein Fd (Shin and Arnon 1965). FNR acts as a transducer between one electron carriers (Fd) and two electron acceptors (NADP$^+$), exploiting the capacity of its prosthetic group (FAD) to be reduced to the semiquinone level by the first electron and then sequentially reduced to the dihydroquinone by the second electron, thus pairing the electrons for hydride transfer to NADP$^+$ (Carrillo and Vallejos 1982; Batie and Kamin 1984):

$$2Fd_{red} + NADP^+ + H^+ \rightarrow 2Fd_{ox} + NADPH$$

In addition to this role in NADP$^+$ photoreduction, FNR is able to catalyze *in vitro* the oxidation of NADPH by suitable electron acceptors like potassium ferricyanide (diaphorase activity) or Fd_{ox} couple to cytochrome *c* reduction.

Function of ferredoxin-NADP$^+$ oxidoreductase in the cyclic photosynthetic electron transport system

Photooxidation of cytochrome b_6 which is a specific component of cyclic electron flow (Böhme and Cramer 1972) appears to involve a number of components common to both cyclic and

linear electron transport (Böhme 1975). Purified FNR showed NADPH-cytochrome *f* reductase activity (Zanetti and Forti 1969) and moreover, addition of an antibody against FNR inhibited cyclic electron photophosphorylation in intact chloroplasts (Forti and Zanetti 1969). The studies using antibodies against spinach Fd and FNR as specific inhibitors of electron transfer reactions indicated that Fd antibody inhibited cytochrome b_6 photoreduction, but the FNR antibody had no effect on cytochrome b_6 reduction (Böhme 1977). These results indicate that FNR is likely to involve in cyclic electron flow around PS I and points to a central role for the enzyme in the modulation photosynthetic electron flow. Therefore, FNR may play a key role in the regulation of cyclic/linear electron flow and then modify the NADPH/ATP ratio in chloroplasts (Mills et al. 1979).

Relationship between ferredoxin-NADP$^+$ oxidoreductase and photosystem I

FNR was believed to be bound on the stromal side of the thylakoid membrane in the vicinity of PS I (Böhme 1977; Carrillo and Vallejos 1982), because FNR bound to Fd (Zanetti and Merati 1987; Zanetti et al. 1988) and represented the branching point between linear and cyclic electron transport. Two pools of FNR appeared to exist *in vivo*, a loosely bound pool which was easily removed from the membrane by a low salt wash and a more tightly bound pool (30-60% of the total enzyme) which required several extensive low salt/EDTA washes and/or addition of detergents (e.g. 3-(3-cholamidopropyl) dimethylammonio-1-propanesulphonate) for its removal (Matthijs et al. 1986). Two distinct FNR pools were postulated, but at present no conclusive data are available demonstrating the existence of two functionally distinct FNR species in photosynthetic tissues. In addition, FNR is mainly located within the nonappressed stromal thylakoid membrane which contains most of the PS I to interact with Fd to transfer electrons for NADP$^+$ reduction.

The PS I is a membrane-bound pigment protein complex which catalyzes light-dependent electron transfer from plastocyanin to Fd. The PS I complex of higher plants contains 12 different polypeptide subunits denoted PS I-A to PS I-L. The isolation of a highly active PS I complex from barley contained the core polypeptide, light-harvesting complex I as well as bound FNR. Cross-linking experiment with 3,3'-dithiobis(sulfosuccinimidyl) propionate and N-ethyl-3-[3-(dimethylamino)propyl]carbodiimide observed a specific interaction between the PS I-E subunit and FNR, suggesting that the PS I-E subunit had an important role in the binding of FNR to the PS I complex (Andersen et al. 1992). On the other hand, 20% of the total FNR was located in the appressed granal lamellae (Vallon et al. 1986), which contain most of the PS II.

Transportation of ferredoxin-NADP$^+$ oxidoreductase into chloroplasts

FNR is encoded in nuclear genome and in common with other nuclear-encoded chloroplast proteins, it is synthesized in the cytoplasm as a higher molecular weight precursor and imported into chloroplasts after translation. The transit peptide is processed for posttranscriptional regulation to the mature protein (Grossman et al. 1982). Molecular chaperones are a class of cellular proteins that play roles in the transport, folding and assembly of certain other polypeptides, but they are not components of the final oligomeric structures (Ellis 1987). The members of the family of Hsp70 were proposed to be molecular chaperons (Gething et al. 1992). Recently, cDNAs encoding a homologue of Hsp70 were isolated in higher plants (Ko et al. 1992; Marshall et al. 1992). Immunoprecipitation studies indicated that pea FNR imported into chloroplasts *in vitro* could interact with antisera raised against the homologue of Hsp70 from pumpkin chloroplasts and against GroEL from *Escherichia coli*, which was a bacterial homologue of Cpn60. These results suggest that homologues of Hsp70 and Cpn60 in chloroplasts may sequentially assist in the maturation of newly imported FNR in an ATP dependent manner (Tsugeki et al. 1993).

ATP dependent post translational modification of ferredoxin-NADP$^+$ oxidoreductase

Protein phosphorylation of thylakoid protein is now a widely established phenomenon (Benett 1984; Ranjeva et al. 1987). Mainly PS II associated proteins were identified as being phosphorylated, including light-harvesting complex II (Benett 1977), D1 and D2 (Marder et al. 1988) and a 9-kDa protein (Hird et al. 1986). It was indicated that FNR was also phosphorylated by the studies incubating with [^{32}P]ATP and purified FNR in pea leaves. Phosphoamino acid analysis using two dimensional electrophoresis showed that FNR could be phosphorylated on a Ser residue in the dark and on Ser and Thr residues in the light (Hodges et al. 1990). The diaphorase activity of phosphorylated FNR was not changed with a preincubation ATP. Perhaps phosphorylation of FNR plays a role in the interaction between FNR and thylakoid membrane, but physiological function is not clear.

Function of specific amino acid residues and three dimensional structures of ferredoxin-NADP$^+$ oxidoreductase

Many experiments using site directed mutagenesis and chemical modification have been reported and the results indicated that specific amino acid residues were essential for FNR function.

In higher plant, the primary structure of FNR has five Cys in the same positions and all of them existed as sulfhydryls (Yao et al. 1985). Spinach FNR has five Cys, four (42, 114, 132, 137) in the FAD binding domain and one (272) in the NADP$^+$ binding domain. The role of the Cys in spinach leaf FNR was investigated by site-directed mutagenesis changing each Cys to Ser. The results indicated that FNR-C42S mutant could not assemble as a holoenzyme. As for the remaining mutants, only FNR-C272S mutant showed an overall decreased catalytic efficiency, whereas FNR-C132S mutant had partially impaired Fd-dependent cytochrome *c* reduction activity but maintained its full diaphorase activity (Aliverti et al. 1993).

The location of the binding site with Fd (Zanetti et al. 1988; Jelesarov et al. 1993), NADP-PPi (Porter and Kasper 1986) and NADP$^+$ (Sheriff and Herriott 1981; Porter and Kasper 1986) were determined and the amino acid residues essential for FNR function were investigated. Chemical modification studies indicated the involvement of Arg at both the NADP$^+$ and Fd binding sites of several FNR species (Zanetti et al. 1979; Bookjans and Böger 1978). Later, the binding sites of spinach FNR for NADP$^+$ and Fd were extensively explored by chemical modification and cross-linking studies. Lys-85 and/or Lys-88 were identified to play a key role in Fd binding as well as, in general, the N-terminal region of the spinach FNR (Zanetti et al. 1988). Lys-116 (Cidaria et al. 1985) and Lys-244 (Chan et al. 1985) were proposed to be involved in NADP$^+$ binding. In *Anabaena* FNR, one Arg was reported to be involved in the interaction with NADP$^+$, while a second such residue was apparently required for the binding with Fd (Sancho et al. 1990).

The C-terminal region of FNR is formed by an invariant α-helixloop/ β-strand and culminating in a conserved Tyr. Orellano et al. (1993) investigated effect of a conserved Tyr by site-directed mutagenesis on pea leaf FNR. The result of the assay for cytochrome *c* reduction indicated that terminal Tyr was essential and its aromaticity was the most important factor to the function of the Tyr in catalysis. The presence of the phenol ring at the C-terminal position of wild-type reductase was important, but not an absolute requirement for enzyme function or FAD assembly.

The three dimensional structure of spinach FNR was first obtained by X-ray crystallography only at low resolution (3.7Å). It revealed that the protein was composed of two domains. The N-terminal half of the polypeptide chain might form the FAD binding

domain and the C-terminal chain should form $NADP^+$ binding domain (Sheriff and Herriott 1981). Ten years later, the three dimensional structure of spinach FNR was determined by X-ray diffraction at 2.6Å high resolution and carried out partial refinement of the model at 2.2Å resolution (Karplus et al. 1991). The results showed that the FAD binding domain (residues 19 to 161) had an antiparallel β barrel core and a single α helix for binding the pyrophosphate of FAD. The $NADP^+$ binding domain (residues 162 to 314) had a central five-strand parallel β sheet and six surrounding helices.

Purification and the amino acid sequence of ferredoxin-$NADP^+$ oxidoreductase protein in photosynthetic tissues

FNR was first purified from spinach (Shin et al. 1963; Shin 1971) and subsequently isolated from other higher plants as well as from eukaryotic algae (Bookjans et al. 1979) and from cyanobacteria (Susor et al 1966; Rowel et al 1981; Javier et al. 1988). All FNRs from the various sources were found to contain FAD as the redox-active coenzyme. The amino acid sequences of the purified FNR were determined from *Spirulina platensis* (Yao et al. 1984) and spinach (Karplus et al. 1984).

Molecular cloning of ferredoxin-$NADP^+$ oxidoreductase cDNA and gene and homology of the deduced amino acid sequence

The cDNA sequence of precursor FNR was determined for the enzymes from spinach (Jansen et al. 1988), pea (Newman and Gray 1988), ice plant (Michalowski et al. 1989), *Cyanophora paradoxa* (Jakowitsch et al. 1993), *Chlamydomonas reinhardtii* (Kitayama et al. 1994), *Arabidopsis thaliana* (Ida et al., unpublished) and broad bean (Lax and Cary, unpublished, Accession No. U14956). On the other hand, the sequence of the FNR structural gene was determined directly from *Anabaena* sp. PCC 7119 (Fillat et al. 1990; 1993), *Synecchococcus* sp. PCC 7002 (Schluchter and Bryant 1992) and *Synechocystis* sp. (Thor Van , unpublished, Accession No. X94297).

The amino acid sequences of the isolated FNR from *Spirulina platensis* (Yao et al. 1984) and spinach (Karplus et al. 1984) are found to have 55% identity. Comparison of the deduced amino acid sequences of FNRs shows that homology of the mature protein of higher plants is more than 80% identity, but homology of FNRs in higher plants to the enzymes from

eukaryotic algae and cyanobacteria is low identity (approximately 50% identity) (See Table 3-1).

Gene expression and regulation of ferredoxin-NADP$^+$ oxidoreductase

Little is known regarding regulation of the expression of FNR gene in photosynthetic tissues. Recently a genomic DNA segment encoding spinach leaf FNR including a 3.4 kb promoter sequence was isolated and partial nucleotide sequences of the clone (-811/+756) were determined. Analysis of the promoter region in GUS gene fusions in transgenic tobacco demonstrated that two light-responsible elements were located within the first 753 bp. The first light-responsible region was located within the first 118 bp upstream of the transcription initiation site and the other was the -220/-119 promoter fragment, which was capable of conferring light-dependent GUS gene expression on two different minimal promoters. The latter fragment bound a transacting factor in gel-shift assays, but the function of the factor are still unknown (Oelmüller et al. 1993).

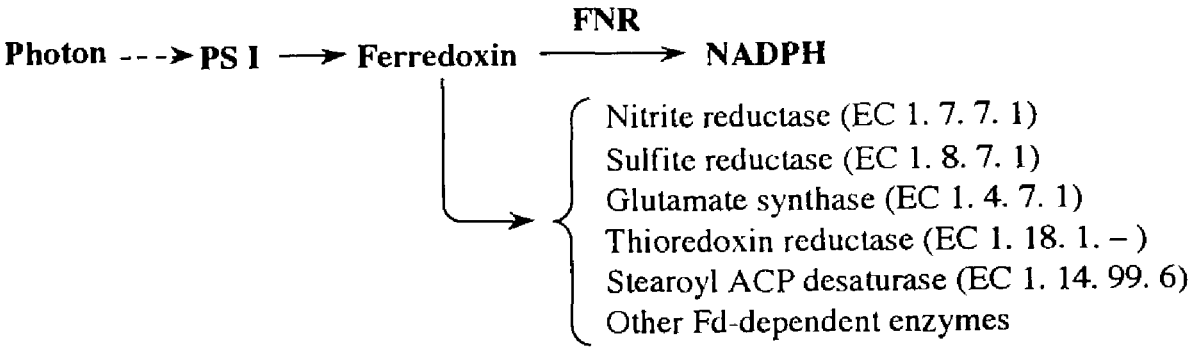
Ferredoxin-NADP$^+$ oxidoreductase and ferredoxin in nonphotosynthetic tissues

In photosynthetic tissues, the nitrogen assimilation enzymes such as NiR and GOGAT located in chloroplasts require Fd_{red} as an electron donor.

On the other hand, several investigations showed that NiR and GOGAT were located in nonphotosynthetic tissues as well as chloroplasts of photosynthetic tissues (Dalling et al. 1972; Oaks and Hirel 1985) and they were found in proplastids from tobacco cultured cell using methyl viologen as an electron donor (Washitani and Sato 1977a; b).

In nonphotosynthetic tissues, the enzymes of the OPPP are located in plastids. It was demonstrated that the OPPP acted as the source of reductant for Fd-dependent enzymes (Emes and Fowler 1979; 1983; Suzuki et al. 1985). The close relationship between nitrite reduction and the OPPP in pea root plastids was confirmed (Bowsher et al. 1989). However these enzymes could not utilize directly NADPH produced by the OPPP (Bowsher et al. 1988; Hucklesby et al. 1972). A key regulatory point for the relationship of them may be the energy intensive reduction of Fd required for NiR and GOGAT activities (Oaks and Hirel 1985). Oji et al. (1985) demonstrated that an electron carrier and a diaphorase activity (FNR activity) were involved in the electron transfer from NADPH to nitrite in plastids from barley roots.

Photosynthetic tissues / organs (leaves)



Nonphotosynthetic tissues / organs (roots and embryos)

Oxidative pentose phosphate pathway

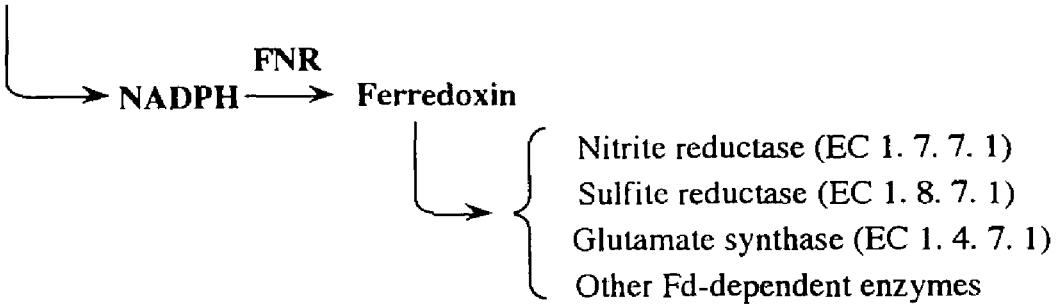


Figure 1-1. Electron transfer system for Fd-dependent enzymes in higher plants.

These results suggested that FNR in nonphotosynthetic tissues catalyzes electron transfer from NADPH to Fd_{ox} and Fd_{red} acts as an electron donor to Fd-dependent enzymes (Figure 1-1) (Chang et al. 1991; Hirasawa et al. 1990: Morigasaki et al. 1990c; 1993). This is in contrast to FNR in photosynthetic tissues where Fd_{red} generated from PS I reduces $NADP^+$ (Knaff and Hirasawa 1991).

Ferredoxin in nonphotosynthetic tissues

It was shown that higher plants have both tissue-specific and nonspecific Fd isoproteins in photosynthetic tissues and that the relative abundance of the isoproteins was regulated by light and stage of development (Wada et al. 1985; Green et al. 1991). The complete amino acid sequences of purified Fds from roots of radish (Wada et al. 1989) and the deduced amino acid sequence of a maize root Fd cDNA (Hase et al. 1991) have been determined and compared with their leaf counterparts, suggesting that they are distinct from the Fds in photosynthetic tissues.

It was shown that each unique Fd was present in the different tissues and its expression seemed to depend on developmental stage as well as environmental conditions (Wada et al. 1985; Kimata and Hase 1989). Studies from several reports revealed that Fds of bean sprouts (Hirasawa et al. 1988), radish roots (Wada et al. 1989), mesophyll and bundle sheath cells in maize leaf (Kimata and Hase 1989) and spinach roots (Morigasaki et al. 1990a) showed that the electron transport activity by monitoring Glu formation and physical properties such as absorption spectrum of root Fd were similar to those of leaf Fd (Morigasaki et al. 1990a).

Purification of ferredoxin-$NADP^+$ oxidoreductase from nonphotosynthetic tissues

A nonphotosynthetic FNR was first purified from rice embryos (Ida and Morita 1970a; 1970b) and it was demonstrated that the absorption spectrum of rice embryo FNR was different from that of FNR from photosynthetic tissues.

The studies of FNRs in radish (Morigasaki et al. 1990b) and spinach roots (Morigasaki et al. 1990a) demonstrated that the root enzymes resemble their leaf counterparts in activity, spectral properties and complex formation, but they differ in amino acid compositions and N-terminal sequences. FNR was also purified from tomato leaves and roots and their cytochrome *c* reduction activity and diaphorase activity were characterized. The results indicated that root FNR had a twice higher cytochrome *c* reduction activity, but a somewhat lower diaphorase activity than the leaf counterpart (Green et al. 1991).

Immunotitration studies of radish root and leaf FNRs by each antiserum showed that the anti-root FNR antiserum effectively inhibited the activity of radish root FNR, but not that of the leaf enzyme and the anti-leaf FNR antiserum strongly inhibited leaf FNR. Root FNR was slightly inhibited by the anti-leaf FNR antiserum but only when a large amount of the antiserum was added. And immunoblot analysis of each FNR showed that leaf FNR was detected only in leaves, but it was not clear whether root FNR was located in leaves because the anti-root FNR antiserum cross-reacted slightly with purified leaf FNR in radish (Morigasaki et al. 1993).

Two FNR isoforms were purified from the first foliage leaves of mung bean (*Vigna radiata*) seedlings (Jin et al. 1994). Immunoblot analysis and N-terminal amino acid sequences showed that one form resembles FNR purified from photosynthetic tissues of higher plants and the other resembled to that from nonphotosynthetic tissues. The studies suggest that leaf FNR is specifically expressed in leaf, whereas root FNR is a nonspecialized form which is also expressed in photosynthetic tissues of young plants and disappeared from leaves when plants mature.

The nitrate assimilation systems in higher plants

Nitrate assimilation is an energy intensive and highly regulated process. Nitrate exposure causes a rapid and transient increase in the accumulation of mRNA transcripts encoding NR (Melzer et al. 1989), NiR (Kramer et al. 1989), GS and GOGAT (Redinbaugh and Campbell 1993; Crawford and Arst 1993). The primary response to nitrate include only those enzymes which act directly in the assimilation of nitrate into amino acids. Several other classes of transcript might be expected to be expressed in the primary response. For example, the rapid and transient accumulation of a transcript encoding a nitrate transporter in *Arabidopsis* exposed to nitrate, suggests that nitrate transporter genes might also be expressed in the primary response to nitrate (Tsay et al. 1993).

Transcription factors for nitrate induction in higher plants

The nitrogen regulatory systems have been studied extensively in lower eukaryotes, for example, in *Neurospora crassa* and *Aspergillus nidulans.*

Fungi have provided invaluable guides for studying nitrate assimilation in plants. Just as in microorganisms, nitrate is actively transported into plant cells via nitrate transporter (Tsay et al. 1993) and reduced to nitrite by NR which is a metalloflavoenzyme containing Mo cofactor. Nitrite was then reduced to ammonium by NiR. Much is known about the structure, function and regulation of the NR and NiR structural genes and the enzymes that they encode in higher plants (Crawford et al. 1992; Hoff et al. 1992; Pelsy and Caboche 1992; Solomonson and Barber 1990). The NR and NiR genes are induced by nitrate and respond to many other signals including light and CO_2, but they do not display the classical ammonium repression seen to fungi.

Recently, nitrate regulatory gene, *nit-2* (a regulatory gene of *N. crassa*) of *Chlamydomonas* , was identified and shown to be necessary for NR gene expression and to be repressed itself by ammonium (Fernandez et al. 1989; Schnell and Lefebvre 1993). In higher plants, a DNA fragment encoding NIT2-like protein was isolated from tobacco (*Nicotiana tabaccum*) by direct PCR method using zinc finger region of *nit-2* gene of *N. crassa* as 5' and 3' primers and subsequently, a cDNA was cloned using the fragment as a probe (Daniel-Vedele and Caboche 1993). The clone, named Ntl1-Nt7, encoded a protein of 305 amino acid residues and contained a single Cys-X_2-Cys-X_{17}-Cys-X_2-Cys type zinc finger DNA binding

motif which was similar to the NIT2 protein. The studies of these genes shall not only advance the understanding of nitrate assimilation, but also provide useful tools for genetic engineering in plants.

As a first step toward studies of nonphotosynthetic FNR cDNA, I report here the isolation and characterization of FNR cDNA in rice roots and the suggestion of involvement of root FNR in the nitrate assimilation pathway. I have also isolated a FNR cDNA from rice leaves. It was reported that the absorption spectrum of rice embryo FNR is different from those of root and leaf FNR, suggesting that an unique FNR is expressed in rice embryo (Ida and Morita 1970a; 1970b). I have isolated a rice embryo FNR cDNA which is neither leaf nor root enzyme in gene structure. Genomic Southern hybridization analysis suggested that both leaf, root and embryo FNR gene was single copy gene, respectively.

Comparison of the deduced amino acid sequences of rice root, embryo and leaf FNR indicated that there are extensive homologies (90% identity) between root and embryo FNRs, whereas leaf FNR has only 49% identity with the root and embryo enzymes. Identical amino acid residues have been indicated to be involved in the binding to FAD, Fd and $NADP^+$. Analysis of the phylogenetic tree and homology of the deduced amino acid sequences suggested that FNRs from higher plants can be divided into two groups, photosynthetic and nonphotosynthetic FNRs. Although the amino acid identities of photosynthetic and nonphotosynthetic FNR are more than 80% within each group, the homology between them are less than 50%. The results suggest that rice leaf FNR and rice root as well as rice embryo FNR belong to evolutionary distinct groups.

Root FNR mRNA was accumulated rapidly after the addition of nitrate to rice seedlings. These results demonstrate that the FNR transcript is induced by nitrate in rice roots. Close similarities of the general patterns of induction of the FNR transcript to those of NR and NiR in root tissues suggest the root FNR is involved in the nitrate assimilation systems. On the other hand, leaf FNR mRNA was accumulated rapidly by exposure of light to rice seedlings. Such light dependency of the leaf enzymes suggests that the regulatory systems differ between photosynthetic and nonphotosynthetic tissues.

The genomic clone corresponding to the rice root FNR cDNA was isolated and sequenced. The rice root FNR gene consists of 6 exons interrupted by 5 introns. The 5' upstream region of the FNR gene has seven GATA-boxes and three TCC..GGA domains, which are specific binding sites to the transcription factors, NIT2 and NIT4 of *N. crassa*. EMSA experiments indicated that there are some proteins in the nuclear extracts from both rice

leaves and roots, which interact with these specific regions of the 5' upstream region of the root FNR gene, suggesting possible occurrence of NIT2- and NIT4-like proteins in rice. Absence of the interaction of the promoter region lacking GATA and TCC..GGA sequences with both nuclear extracts from rice leaves and roots suggest those specific sequences are possibly involved in the binding to the nuclear proteins in rice.

CHAPTER II

CLONING AND CHARACTERIZATION OF cDNAs ENCODING LEAF, ROOT AND EMBRYO FERREDOXIN-NADP$^+$ OXIDOREDUCTASES FROM RICE

II-1 Molecular cloning of a cDNA encoding rice leaf ferredoxin-NADP$^+$ oxidoreductase

FNR has been isolated and extensively characterized from a number of plant, algal and cyanobacterial sources (Knaff and Hirasawa 1991; Knaff 1996). The enzyme's amino acid sequence has been described for spinach (Karplus et al. 1984; Jansen et al. 1988), pea (Newman and Gray 1988) and ice plant (Michalowski et al. 1989) as well as other photosynthetic organisms. However, the primary sequences from higher plant FNRs are limited to those from the dicots.

I have isolated a full-length cDNA clone encoding the rice leaf enzyme and sequenced it. I report in this Chapter the complete nucleotide sequence of a rice leaf FNR cDNA.

Materials and Methods

Purification of rice leaf FNR

Leaf FNR was purified to homogeneity by butyl-Toyopearl and Fd-Sepharose chromatography (Shin et al. 1990) for an FNR fraction obtained during the purification of rice leaf nitrite reductase (Ida et al. 1989).

Determination of the amino acid sequences for N-terminal region of the mature protein

Amino acid sequencing was performed according to Hirano (1993). SDS-PAGE of purified FNR was performed on 10% polyacrylamide gels. The enzyme was transferred to a PVDF membrane presoaked in methanol and stained with 0.5% Ponceau S in 1% acetic acid for 5 min. After staining, the membrane was washed with destaining solution (20% methanol/ 5% acetic acid) until clear background appeared. The membrane was washed again in water for 5 min

and dried up overnight. A red band corresponding to 35 kDa was cut out and subjected directly to peptide sequencing for the N-terminal amino acid sequence by the automated Edman degradation with a model 477A gas phase sequencer of Applied Biosystems.

Screening of the cDNA library

A rice leaf cDNA library constructed in lambda gt 11 from poly(A)$^+$ RNA of nitrate-induced greening rice seedlings (Terada et al. 1995) was used for screening and cloning of leaf FNR cDNA. The library was immunoscreened with an antiserum raised against rice leaf FNR (Aoki et al. 1994).

Determination of the nucleotide sequences

The positive clone L9 was digested with *Eco* RI and subsequently the 1.4 kb insert was subcloned into pBS SK+ (Stratagene) according to Hayashi et al.(1986) using a Takara DNA ligation kit and transformed into *E. coli* strain MV1184. The cDNA insert was digested with several restriction enzymes and short inserts were recloned into pBS SK+. For inserts larger than 0.4 kb, they were subjected to nested deletion according to Henikoff (1984) using a Takara kilo-sequence deletion kit. For this purpose, the plasmid DNA was cleaved with either *Apa* I and *Eco* RI or *Eco* RI and *Sac* II. The DNA was digested with exonuclease III for 0.5 to 5 min at 37 °C and trimmed with mung bean nuclease for 1 h at 37 °C. The ends were rendered blunt with Klenow enzyme and religated to circularize deletion subclones. Both strands of the cDNA were sequenced by the dideoxy chain termination method with an Applied Biosystems model 373A DNA sequencers with use of *Taq* DNA polymerase and -21M13 and M13RP primers (Sanger et al. 1977).

Computer methods

Sequence analysis, M_r, GC content, pI and maximum homology (%) were performed by DNASIS-Mac v3.5 program (Hitachi Software Engineering Co., Ltd.). Homology alignment was done with the Clustal W program.

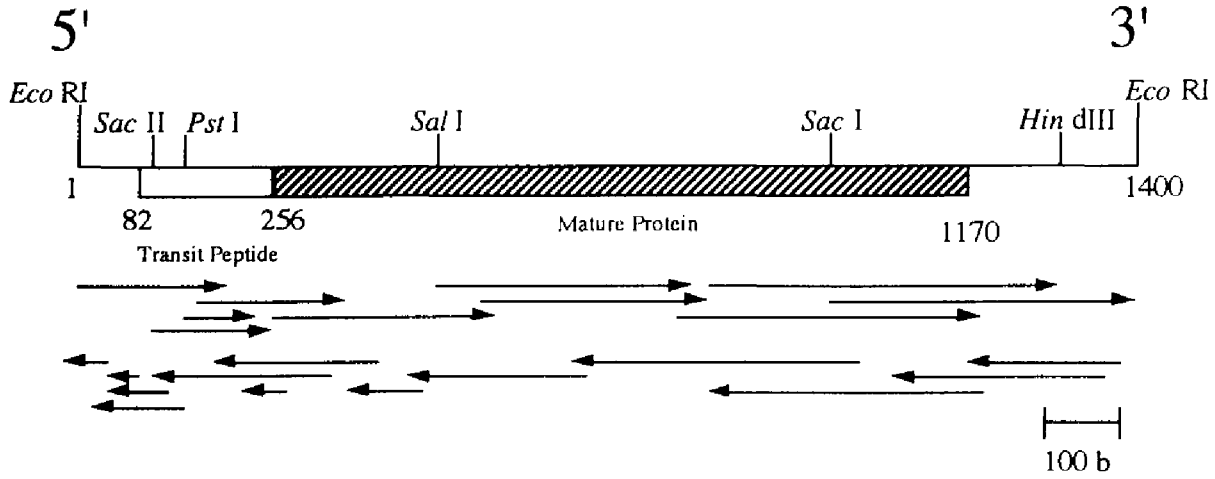


Figure 2-1. Restriction map and strategy of sequencing of the rice leaf FNR cDNA clone. The cross-hatched bar represents the mature protein, the white bar indicates the transit peptide. The sequencing strategy is outlined with arrows.

Results and Discussion

The first immunoscreening of the cDNA library provided a large number of positive plaques. After second screening several positive clones were purified and the longest clone (L9) with a 1.4 kb insert was chosen for sequencing. The insert of the clone L9 was digested with *Eco* RI and subcloned into pBS SK+. A restriction map of this clone and the strategy used for sequencing are shown in Figure 2-1. There is one *Hin* dIII (1302), *Pst* I (125), *Sac* I (1003), *Sac* II (98) and *Sal* I (472) restriction enzyme site in this clone. The L9 insert was cleaved with these restriction enzymes and short inserts were recloned into pBS SK+. In addition, deletion subclones of the leaf FNR cDNA clone were made and all above clones were sequenced.

Complete nucleotide sequence and the deduced amino acid sequence of the rice leaf FNR cDNA clone (L9) are displayed in Figure 2-2. The calculated GC content is 52.8%. The cDNA is 1,400 bp long and carries an ORF of 1086 bp and a 81 bp 5' and a 233 bp 3' noncoding regions (Figure 2-2). The N-terminal 12 residues of purified rice leaf FNR was sequenced. The amino acid sequence, AAAPAKKEKISK, is revealed as shown in Figure 2-2. In the 3' untranslated region, there are two possible polyadenylation signals, AATAAT, to which the same hexanucleotide lies consecutively.

The first 58 amino terminal stretch is assigned as a putative transit peptide, as the N-terminus of the isolated protein starts with Ala at position 59. A molecular mass of the deduced mature protein is 34,795, the value being in excellent agreement with 35.0 kDa estimated on SDS-PAGE for the isolated enzyme. Although it was demonstrated that transit peptides were

```
   1 gaattcgcggccgcctcctcctcacacctgcacacacttgcacacttgca   50
  51 cccaccctctctcctccatccagcaccgaccATGGCCGCCGTCACGGCCG  100
   1                                  M  A  A  V  T  A      6
 101 CGGCCGTCTCCACCTCCGCCGCTGCTGCAGTCACCAAGGCATCGCCGTCC  150
   7 A  A  V  S  V  S  A  A  A  A  V  T  K  A  S  P  S   23
 151 CCCGCCCACTGCTTCCTGCCATGCCCGCCAAGAACCAGAGCCGCCCACCA  200
  24  P  A  H  C  F  L  P  C  P  P  R  T  R  A  A  H  Q   40
 201 GCGCGGCCTGCTGCTGCGCGCGCAGGTGTCCACCACCGACGCCGCCGCCG  250
  41   R  G  L  L  L  R  A  Q  V  S  T  T  D  A  A  A    56
 251 TCGCCGCCGCGCCGGCCAAGAAGGAGAAGATATCCAAGAAGCATGACGAG  300
  57 V  A  A  A  P  A  K  K  E  K  I  S  K  K  H  D  E   73
 301 GGCGTCGTCACCAACAAGTACAGGCCCAAGGAGCCCTACGTCGGCAAGTG  350
  74  G  V  V  T  N  K  Y  R  P  K  E  P  Y  V  G  K  C   90
 351 CCTCCTCAACACCAAGATCACCGCCGACGACGCGCCCGGCGAGACATGGC  400
  91   L  L  N  T  K  I  T  A  D  D  A  P  G  E  T  W    106
 401 ACATGGTCTTCAGCACCGAGGGTGAGATCCCCTACAGAGAGGGGCAGTCC  450
 107 H  M  V  F  S  T  E  G  E  I  P  Y  R  E  G  Q  S   123
 451 ATCGGCGTCATCGCCGACGGCGTCGACAAGAACGGCAAGCCGCACAAGCT  500
 124  I  G  V  I  A  D  G  V  D  K  N  G  K  P  H  K  L   140
 501 CAGGCTCTACTCCATCGCCAGCAGCGCTCTCGGCGACTTCGGCGACTCCA  550
 141   R  L  Y  S  I  A  S  S  A  L  G  D  F  G  D  S    156
 551 AGACCGTTTCACTCTGCGTCAAGAGGCTCGTTTACACCAACGACCAGGGA  600
 157 K  T  V  S  L  C  V  K  R  L  V  Y  T  N  D  Q  G   173
 601 GAGATTGTCAAAGGAGTCTGCTCCAACTTCCTCTGTGACTTGAAGCCTGG  650
 174  E  I  V  K  G  V  C  S  N  F  L  C  D  L  K  P  G   190
 651 TTCTGATGTCAAGATAACCGGACCAGTAGGCAAAGAAATGCTCATGCCCA  700
 191   S  D  V  K  I  T  G  P  V  G  K  E  M  L  M  P    206
 701 AAGATCCCAATGCTAATATTATAATGCTTGCGACCGGTACTGGTATTGCC  750
 207 K  D  P  N  A  N  I  I  M  L  A  T  G  T  G  I  A   223
 751 CCGTTCCGCTCATTCTTGTGGAAAATGTTTTTTGAGAAGTATGATGACTA  800
 224  P  F  R  S  F  L  W  K  M  F  F  E  K  Y  D  D  Y   240
 801 CAAGTTCAATGGTCTGGCTTGGCTCTTCTTGGGAGTCCCAACTAGCAGTT  850
 241   K  F  N  G  L  A  W  L  F  L  G  V  P  T  S  S    256
 851 CTTTACTCTACAAGGAGGAGTTTGACAAAATGAAGGCGAAAGCGCCAGAG  900
 257 S  L  L  Y  K  E  E  F  D  K  M  K  A  K  A  P  E   273
 901 AACTTCCGGGTCGATTATGCTGTGAGCAGGGAGCAGACCAATGCTCAAGG  950
 274  N  F  R  V  D  Y  A  V  S  R  E  Q  T  N  A  Q  G   290
 951 AGAGAAGATGTACATTCAGACCAGGATGGCAGAGTACAAGGAAGAGCTGT 1000
 291   E  K  M  Y  I  Q  T  R  M  A  E  Y  K  E  E  L    306
1001 GGGAGCTCCTGAAGAAGGACCACACCTATGTGTACATGTGTGGACTGAAA 1050
 307 W  E  L  L  K  K  D  H  T  Y  V  Y  M  C  G  L  K   324
1051 GGCATGGAGAAGGGTATTGATGACATTATGGTGTCATTGGCTGCAAAAGA 1100
 324  G  M  E  K  G  I  D  D  I  M  V  S  L  A  A  K  D   340
1101 TGGAATCGACTGGGCTGATTACAAGAAGCAACTGAAGAAGGGCGAGCAAT 1150
 341   G  I  D  W  A  D  Y  K  K  Q  L  K  K  G  E  Q    356
1151 GGAACGTGGAAGTCTACTAAttcttccaattttcctcacatctgtttctt 1200
 357 W  N  V  E  V  Y  *                                  362
1201 ttttttcttccatttgtatctgtgtgcacatctgtgcctgtgatcactct 1250
1251 ataatgttagataggcgtatatatatactgtttgtcatgttggttaaatt 1300
1301 caagcttcatataagaattactacttatgtctgatccaaatactactatg 1350
1351 gtcaagtcaagagtaataataataataatgcaatgcgcggccgcgaattc 1400
```

Figure 2-2. Nucleotide and the deduced amino acid sequences of the rice leaf FNR cDNA. Sequence in lowercase letters indicates the untranslated region and capital letters represent the coding sequence. Italic capital letters represent the deduced amino acid sequence. Possible polyadenylation signals are double-underlined. The termination codon (TAA) is marked with an asterisk (*). The N-terminal sequence of the purified protein is underlined.

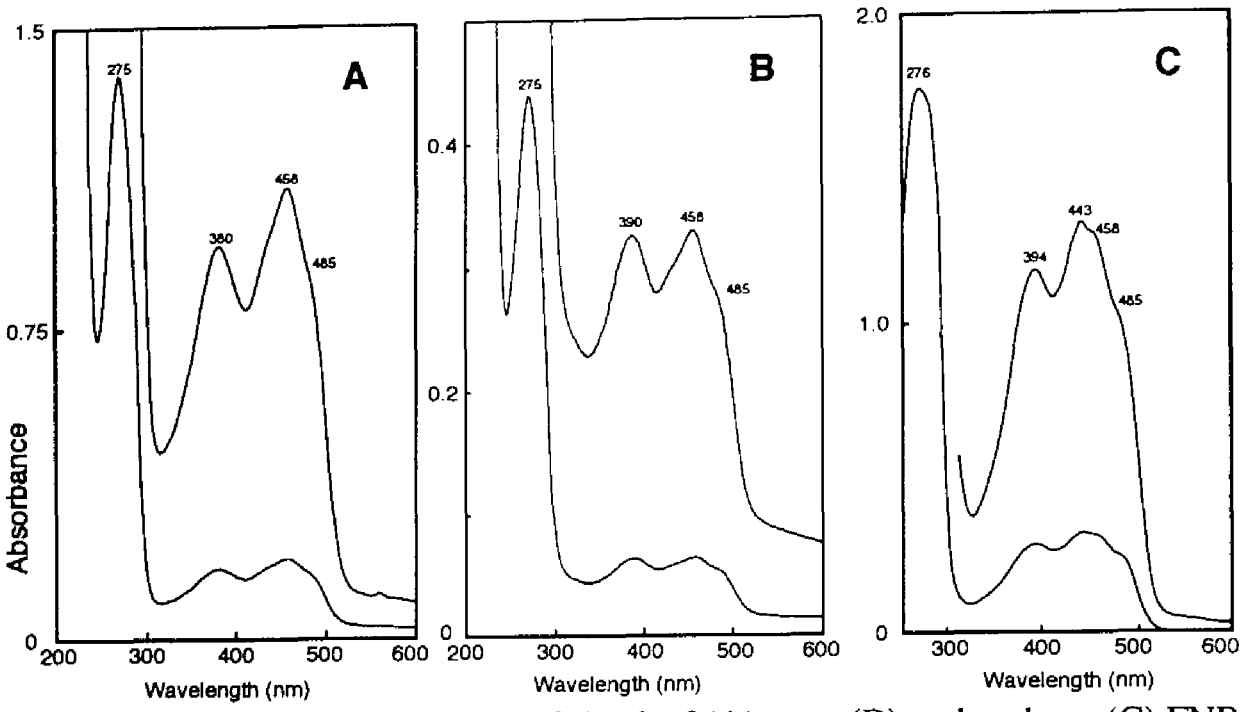


Figure 2-3. Absorption spectra of rice leaf (A), root (B) and embryo (C) FNR

necessary and sufficient for uptake and processing by chloroplasts, the signals required for the recognition and import of proteins by chloroplasts were not clearly defined. The transit peptide of rice leaf FNR contains a high proportion of Ala (18 residues) and a single Asp, but no Glu. Such salient features are characteristic of the chloroplast transit peptides (Archer and Keegstra 1990). Although there are a few identical amino acids in the transit peptides, comparison of the predicted amino acid sequences of the deduced mature proteins reveals extensive homology to other photosynthetic FNRs from higher plants (See Chapter III).

II-2 Molecular cloning of a cDNA encoding rice root ferredoxin-NADP$^+$ oxidoreductase

Flavoprotein enzymes with similar properties to photosynthetic FNRs have been purified and characterized from rice embryos (Ida and Morita 1970a; 1970b), roots of radish (Morigasaki et al. 1990b), spinach (Morigasaki et al. 1990c), tomato (Green et al. 1991) and bean sprouts (Hirasawa and Knaff 1990). Ida and Morita (1970a) demonstrated that the absorption spectrum of FNR from rice embryos is different from those of FNRs from photosynthetic tissues (Figure 2-3). Nonphotosynthetic FNRs have been implicated in nitrate assimilation in nonchlorophyllous tissues where the reverse electron transfer from NADPH to Fd via FNR is

thought to occur in order to provide the reducing equivalent for nitrite reduction and glutamate synthesis.

Despite a wealth of information on photosynthetic FNR (Knaff 1996), the structural and genetic aspects of the enzyme from nongreen tissues or organs remain obscure. I describe here molecular cloning and the complete nucleotide sequence of a cDNA encoding rice root FNR.

Materials and Methods

Purification of rice root FNR and determination N-terminal sequence of mature protein

Rice root FNR was purified by the same procedure as used for rice leaf FNR. Purified root FNR was more than 90% pure and its M_r was estimated to be 35.0 kDa on SDS-PAGE. The amino terminal sequence was determined for the protein blotted on a PVDF membrane using an Applied Biosystems 477A protein sequencer as described in Chapter II-1.

Molecular cloning of the rice root FNR cDNA

A rice root cDNA library was constructed in lambda gt 11 from root tissue of greening seedlings that were induced for 90 min with 10 mM nitrate. The cDNA library was immunoscreened using the rice leaf FNR polyclonal antibody by standard procedures (Sambrook et al. 1989). After second screening, several positive clones were obtained and the clones (R2 and R14) carrying 1.4 kb insert were subcloned into pBS SK+ with *Eco* RI site according to Hayashi et al. (1986) using a Takara DNA ligation kit and transformed into *E. coli* MV1184. The cDNA insert was digested with several restriction enzymes and short inserts were recloned into pBS SK+. Deletion subclones were generated according to Henikoff (1984) using a Takara kilo-sequence deletion kit. The R2 and R14 cDNA clones were sequenced for both directions using an Applied Biosystems sequencing kit with *Taq* DNA polymerase and -21M13, M13RP primers based on the dideoxy chain termination method (Sanger et al. 1977).

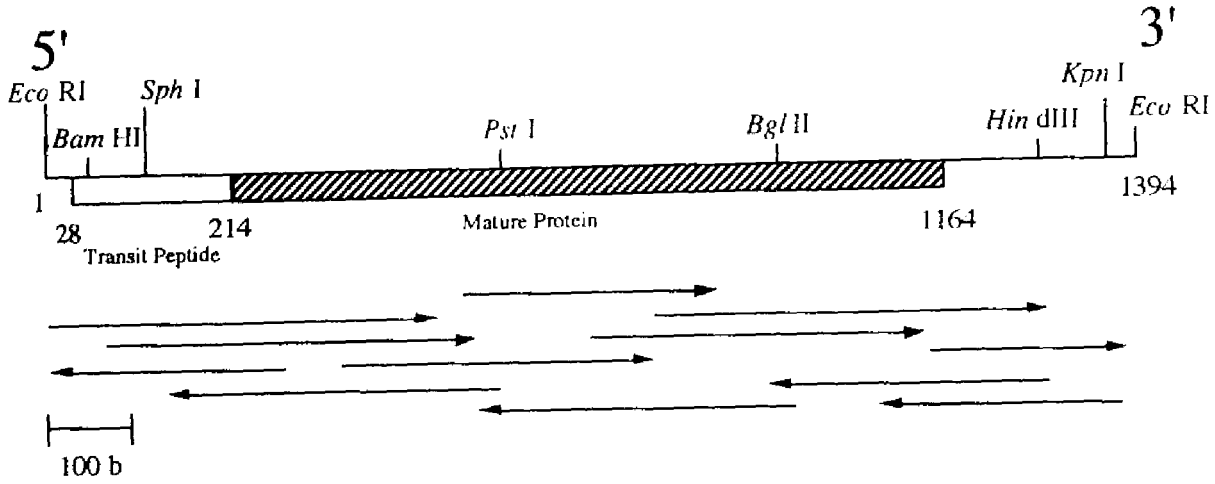


Figure 2-4. Restriction map and strategy of sequencing of the rice root FNR cDNA clone. The cross-hatched bar represents the mature protein, the white bar indicates the transit peptide. The sequencing strategy is outlined with arrows.

Spectral measurements

Absorption spectrum of purified rice root FNR was recorded with a Shimadzu multiple recording spectrophotometer MPF-2.

Results and Discussion

A restriction map of the clone R14 and the strategy for sequencing are shown in Figure 2-4. There is one *Bam* HI (101), *Bgl* II (992), *Hin* dIII (1305), *Kpn* I (1369), *Pst* I (612) and *Sph* I (189) restriction enzyme site in the clone R14. The insert was cleaved with these restriction enzymes and short inserts were recloned into pBS SK+. In addition, deletion subclones were made in both directions and these subclones were sequenced.

Nucleotide and the deduced amino acid sequences of R14 cDNA are shown in Figure 2-5. The calculated GC content is 49.3%. The nucleotide sequence comprises 27 bp 5' noncoding, 1134 bp coding and 233 bp 3' noncoding regions. In the 3' untranslated region, there is a possible polyadenylation signals, ATAAAA. The N-terminal sequence of purified rice root FNR was determined up to the 9th residue. The sequence is revealed to be SVQQASESK, as shown in Figure 2-5. The cDNA consists of 378 amino acids, of which the N-terminal 62 residues is regarded as a transit peptide, because the isolated protein starts with the Ser at position 63. M_r of the deduced mature protein is 35,432, the value being in excellent agreement with 35.0 kDa estimated on SDS-PAGE for the isolated enzyme. Sizes of the

```
   1 gaattcgcggccgcctcaggatcggccATGGCGACCGCCGTTGCGTCCCA   50
   1                          M  A  T  A  V  A  S  Q      8
  51 GGTTGCTGTCTCTGCTCCGGCTGGCTCGGATCGCGGCTTGAGGAGTTCTG  100
   9  V  A  V  S  A  P  A  G  S  D  R  G  L  R  S  S     24
 101 GGATCCAGGGTAGCAACAATATTAGCTTTAGCAACAAATCATGGGTTGGC  150
  25 G  I  Q  G  S  N  N  I  S  F  S  N  K  S  W  V  G   41
 151 ACCACATTGGCGTGGGAGAGCAAGGCCACGCGACCGAGGCATGCGAACAA  200
  42  T  T  L  A  W  E  S  K  A  T  R  P  R  H  A  N  K  58
 201 GGTGCTCTGCATGTCAGTTCAGCAAGCGAGCGAAAGCAAGGTTGCTGTCA  250
  59  V  L  C  M  S  V  Q  Q  A  S  E  S  K  V  A  V    74
 251 AGCCTCTTGATTTGGAGAGTGCTAACGAGCCGCCGCTCAACACATACAAA  300
  75 K  P  L  D  L  E  S  A  N  E  P  P  L  N  T  Y  K   91
 301 CCAAAGGAGCCTTACACCGCCACAATTGTCTCGGTTGAGAGGATCGTAGG  350
  92  P  K  E  P  Y  T  A  T  I  V  S  V  E  R  I  V  G  108
 351 CCCCAAGGCTCCAGGAGAGACATGCCACATTGTTATTGATCATGGTGGCA  400
 109   P  K  A  P  G  E  T  C  H  I  V  I  D  H  G  G   124
 401 ATGTGCCTTACTGGGAGGGGCAAAGCTATGGCATTATTCCTCCAGGGGAG  450
 125 N  V  P  Y  W  E  G  Q  S  Y  G  I  I  P  P  G  E  141
 451 AACCCGAAGAAGCCTGGTGCACCACATAATGTCCGTCTTTATTCAATTGC  500
 142  N  P  K  K  P  G  A  P  H  N  V  R  L  Y  S  I  A  158
 501 ATCTACAAGGTATGGAGATTCATTCGATGGAAGGACCACTAGTTTATGTG  550
 159   S  T  R  Y  G  D  S  F  D  G  R  T  T  S  L  C   174
 551 TGCGCCGTGCCGTTTATTATGATCCTGAAACTGGCAAGGAGGACCCCTCA  600
 175 V  R  R  A  V  Y  Y  D  P  E  T  G  K  E  D  P  S  191
 601 AAAAATGGTGTCTGCAGTAACTTCCTATGTAATTCAAAACCAGGGGACAA  650
 192  K  N  G  V  C  S  N  F  L  C  N  S  K  P  G  D  K  208
 651 GGTTAAAGTGACAGGTCCGTCAGGCAAAATAATGCTCCTGCCTGAGGAAG  700
 209   V  K  V  T  G  P  S  G  K  I  M  L  L  P  E  E   224
 701 ATCCAAATGCAACTCACATCATGATAGCTACTGGCACTGGTGTTGCTCCA  750
 225 D  P  N  A  T  H  I  M  I  A  T  G  T  G  V  A  P  241
 751 TTCCGTGGCTACCTACGCCGTATGTTCATGGAAGATGTCCCAAAGTACAG  800
 242  F  R  G  Y  L  R  R  M  F  M  E  D  V  P  K  Y  R  258
 801 ATTTGGTGGCTTGGCCTGGCTCTTCCTTGGTGTGGCTAACACTGACAGCC  850
 259   F  G  G  L  A  W  L  F  L  G  V  A  N  T  D  S   274
 851 TTCTCTATGATGAAGAGTTCACAAGCTACCTTAAGCAGTATCCAGACAAT  900
 275 L  L  Y  D  E  E  F  T  S  Y  L  K  Q  Y  P  D  N  291
 901 TTCAGGTATGACAAAGCGCTAAGCAGGGAGCAGAAAAACAAGAACGCTGG  950
 292  F  R  Y  D  K  A  L  S  R  E  Q  K  N  K  N  A  G  308
 951 CAAGATGTATGTCCAGGACAAGATCGAGGAGTACAGCGACGAGATCTTCA 1000
 309   K  M  Y  V  Q  D  K  I  E  E  Y  S  D  E  I  F   324
1001 AGCTCTTGGATGGCGGCGCGCACATCTACTTCTGTGGTTTGAAGGGGATG 1050
 325 K  L  L  D  G  G  A  H  I  Y  F  C  G  L  K  G  M  341
1051 ATGCCTGGGATTCAAGACACCCTCAAGAAAGTGGCGGAGCAGAGAGGGGA 1100
 342  M  P  G  I  Q  D  T  L  K  K  V  A  E  Q  R  G  E  358
1101 GAGCTGGGAGCAGAAGCTATCCCAGCTCAAGAAGAACAAGCAATGGCACG 1150
 359   S  W  E  Q  K  L  S  Q  L  K  K  N  K  Q  W  H   374
1151 TTGAGGTCTACTAGgatctaagtgtccaaggattatgattgttgcgcagt 1200
 375 V  E  V  Y  *                                      378
1201 gaaaaagagaaaacaaaacgcatgatctgatgattcttgtagggtggtgt 1250
1251 aaaatcatcatttttttctgaatatgaatcataaaatcacccatgtaat 1300
1301 tcataagcttctgcatcacatgatgaacgaaaggaagcatgtaacttttg 1350
1351 cctgtcactattgcagctggtacctttgctgcggccgcgaattc       1394
```

Figure 2-5. Nucleotide and the deduced amino acid sequences of the rice root FNR cDNA. Sequence in lowercase letters indicates the untranslated region and capital letters represent the coding sequence. Italic capital letters represent the deduced amino acid sequence. Possible polyadenylation signal is double-underlined. The termination codon (TAG) is marked with an asterisk (*). The N-terminal sequence of the purified protein is underlined.

precursor polypeptide and the transit sequence are very close to those of photosynthetic FNRs (Jansen et al. 1988; Newman and Gray 1988; Michalowski et al. 1989). The R2 cDNA clone was also sequenced, but the clone starts from position 47 of the full-length R14 clone, indicating that the R2 clone was a partial FNR cDNA.

After publication of the rice root FNR cDNA sequence, two heterotrophic FNR cDNA clones have been isolated from maize roots (Ritchie et al. 1994) and pea roots (Bowsher and Knight 1996). A comparison of these three root FNRs revealed an extensive homology between them (See Chapter III).

Absorption spectrum of rice root FNR is shown in Figure 2-3. The spectrum is characteristic of flavoproteins, but absorption maxima and shoulders at 390, 458 and 485 nm are slightly different from those of rice leaf FNR at 394, 443, 458, 485 nm shown in Figure 2-3, suggesting that FNR expressed in rice roots has a different amino acid sequence from that of FNR from rice leaves.

II-3 Molecular cloning of a cDNA encoding rice embryo ferredoxin-$NADP^+$ oxidoreductase

The first nongreen flavoprotein enzyme similar to photosynthetic FNR was purified and characterized from rice embryos (Ida and Morita 1970a; 1970b), but its protein sequence remained to be determined. It was reported that the absorption spectrum of rice embryo FNR is different from that of leaf FNR (Figure 2-3), suggesting that different FNR is expressed in rice embryo. I describe in this Chapter an embryo-specific FNR cDNA which is neither leaf nor root enzyme in gene structure.

Materials and Methods

Molecular cloning of the rice root FNR cDNA

A rice embryo cDNA library was constructed in lambda ZAP II (Stratagene) from developing rice seeds 14 days after flowering. The library was a gift from Professor Kunisuke Tanaka, Department of Biochemistry, College Agriculture, Kyoto Prefectural University.

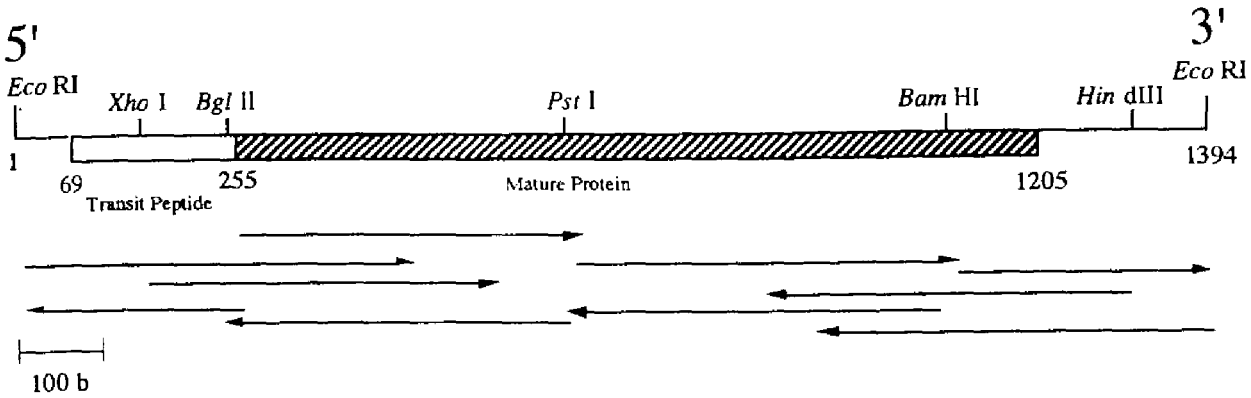


Figure 2-6. Restriction map and strategy of sequencing of the rice embryo FNR cDNA clone. The cross-hatched bar represents the mature protein, the white bar indicates the transit peptide. The sequencing strategy is outlined with arrows.

The cDNA library was screened using a PCR-amplified 875 bp DNA fragment of nucleotides from 289 to 1164 of the rice root FNR cDNA clone as a probe. The probe was labeled and detected with ECL direct nucleic acid labeling and detection systems (Amersham). After second screening, 8 positive phage clones were obtained from which a positive clone (E1) harboring 1.4 kb insert was transformed into pBS SK- in XL1-blue as a host cell by *in vivo* plasmid excision (Short et al. 1988; Short and Sorge 1992). The cDNA insert was digested with several restriction enzymes and short inserts were recloned into pBS SK+. The E1 cDNA clone was sequenced for both directions using an Applied Biosystems sequencing kit with *Taq* DNA polymerase and -21M13, M13RP primers based on the dideoxy chain termination method (Sanger et al. 1977).

Results and Discussion

A restriction map of the E1 clone and the strategy used for sequencing are shown in Figure 2-6. There is one *Bam* HI (1099), *Bgl* II (241), *Hin* dIII (1318), *Pst* I (653) and *Xho* I (140) restriction enzyme site in this clone. The restriction map of the E1 cDNA clone differs from that of the rice root cDNA clone, indicating that the embryo clone carries a different FNR gene. The insert was cleaved with these restriction enzymes and short inserts were sequenced.

The rice embryo cDNA clone was identified as a full-length FNR cDNA clone by sequencing. Nucleotide and the deduced amino acid sequences of the E1 cDNA clone are shown in Figure 2-7. The calculated GC content of E1 is 45.2%. The embryo FNR cDNA consists of 68 bp 5' noncoding, 1134 bp coding and 192 bp 3' noncoding regions. In the 3' untranslated region, there is a putative polyadenylation signals, AATAAA (Figure 2-7).

```
   1 gaattcgcggccgctcaaaaccctagccaaccccctcctcctcctcctctt   50
  51 cctcgcgatccaccggcgATGGCCTCCGCCCTCGGGGCTCAGGCGTCTGT  100
   1                    M  A  S  A  L  G  A  Q  A  S  V    11
 101 CGCGGCGCCCATCGGTGCGGGCGGCTACGGGAGGAGTTCCTCGAGCAAGG  150
  12    A  A  P  I  G  A  G  G  Y  G  R  S  S  S  S  K     27
 151 GTAGCAATACTGTTAACTTCTGCAACAAATCATGGATTGGAACCACATTA  200
  28 G  S  N  T  V  N  F  C  N  K  S  W  I  G  T  T  L     44
 201 GCATGGGAAAGCAAGGCCCTAAAATCAAGGCATATGAACAAGATCTTTTC  250
  45  A  W  E  S  K  A  L  K  S  R  H  M  N  K  I  F  S    61
 251 CATGTCCGTTCAACAAGCAAGCAAAAGCAAAGTTGCTGTAAAACCTCTGG  300
  62    M  S  V  Q  Q  A  S  K  S  K  V  A  V  K  P  L     77
 301 AATTGGATAATGCGAAGGAGCCACCCCTTAACTTATACAAACCAAAGGAG  350
  78 E  L  D  N  A  K  E  P  P  L  N  L  Y  K  P  K  E     94
 351 CCTTACACAGCCACAATTGTCTCAGTCGAAAGGCTTGTAGGCCCTAAAGC  400
  95  P  Y  T  A  T  I  V  S  V  E  R  L  V  G  P  K  A   111
 401 TCCTGGTGAAACATGCCATATTGTTATTGATCATGGTGGCAATGTTCCAT  450
 112    P  G  E  T  C  H  I  V  I  D  H  G  G  N  V  P    127
 451 ACTGGGAAGGACAAAGTTATGGTGTCATTCCTCCAGGAGAGAACCCGAAG  500
 128 Y  W  E  G  Q  S  Y  G  V  I  P  P  G  E  N  P  K    144
 501 AAACCTGGTTCCCCAAATACTGTCCGGCTCTATTCTATTGCATCTACTAG  550
 145  K  P  G  S  P  N  T  V  R  L  Y  S  I  A  S  T  R   161
 551 GTACGGTGATTCTTTTGATGGAAAGACTGCCAGTTTGTGTGTTCGTCGTG  600
 162    Y  G  D  S  F  D  G  K  T  A  S  L  C  V  R  R    177
 601 CTGTTTATTATGATCCTGAAACTGGAAAAGAAGACCCCACAAAGAAAGGT  650
 178 A  V  Y  Y  D  P  E  T  G  K  E  D  P  T  K  K  G    194
 651 ATCTGCAGTAATTTCCTATGCGACTCTAAACCAGGCGACAAAGTTCAGAT  700
 195  I  C  S  N  F  L  C  D  S  K  P  G  D  K  V  Q  I   211
 701 AACAGGCCCCTCAGGCAAAATCATGCTTCTACCTGAGGATGATCCAAATG  750
 212    T  G  P  S  G  K  I  M  L  L  P  E  D  D  P  N    227
 751 CAACCCATATCATGATTGCTACTGGCACTGGTGTTGCTCCCTACCGTGGC  800
 228 A  T  H  I  M  I  A  T  G  T  G  V  A  P  Y  R  G    244
 801 TATCTACGTCGTATGTTCATGGAGGATGTCCCAAGTTTCAAGTTTGGTGG  850
 245  Y  L  R  R  M  F  M  E  D  V  P  S  F  K  F  G  G   261
 851 TCTGGCTTGGCTATTTCTAGGTGTTGCTAACACTGATAGCCTTCTGTATG  900
 262    L  A  W  L  F  L  G  V  A  N  T  D  S  L  L  Y    277
 901 ATGAAGAGTTCACAAACTACCTTCAGCAGTATCCAGACAATTTCAGGTAT  950
 278 D  E  E  F  T  N  Y  L  Q  Q  Y  P  D  N  F  R  Y    294
 951 GATAAAGCACTAAGTAGGGAACAGAAGAATAAGAATGGTGGAAAGATGTA 1000
 295  D  K  A  L  S  R  E  Q  K  N  K  N  G  G  K  M  Y   311
1001 TGTGCAGGACAAGATTGAAGAGTACAGCGATGAAATTTTTAAACTTTTGG 1050
 312    V  Q  D  K  I  E  E  Y  S  D  E  I  F  K  L  L    327
1051 ATGGCGGTGCACATATCTACTTTTGTGGTTTGAAGGGTATGATGCCAGGG 1100
 328 D  G  G  A  H  I  Y  F  C  G  L  K  G  M  M  P  G    344
1101 ATCCAGGACACACTCAAGAGAGTAGCTGAGCAAAGAGGTGAGAGTTGGGA 1150
 345  I  Q  D  T  L  K  R  V  A  E  Q  R  G  E  S  W  E   361
1151 GCAGAAGCTGTCGCAGCTCAAAAAGAACAAACAATGGCACGTGGAGGTTT 1200
 362    Q  K  L  S  Q  L  K  K  N  K  Q  W  H  V  E  V    377
1201 ACTAAgttactaaaaagcacgggctgtgattttgtgattgttttgcagcg 1250
 378 Y  *                                                378
1251 agttgaaacataaaacagtaaaaagcgatgattctcgttgcattgtaaaa 1300
1301 ttgtcaatcttattcataagcttctgcttgacatggtgaataaaatgaag 1350
1351 catatgctaattttgacttaaaaaaaaaaagcggccgcgaattc       1394
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Figure 2-7. Nucleotide and the deduced amino acid sequences of the rice embryo FNR cDNA. Sequence in lowercase letters indicates the untranslated region and capital letters represent the coding sequence. Italic capital letters represent the deduced amino acid sequence. Possible polyadenylation signal is double-underlined. The termination codon (TAA) is marked with an asterisk (*). The N-terminal sequence of the purified protein is underlined.

The coding region encodes 378 amino acid residues. Since the ORF contains the same sequence, SVQQAS, as that of the rice root FNR mature protein, the first N-terminal 62 amino acid residues were assigned as a putative transit peptide. The deduced M_r of the mature protein is 35,407. Sizes of the embryo precursor polypeptide and the transit sequence are the same to those of rice root FNR. pI of rice embryo FNR mature protein is 7.68.

Although the physiological function of rice embryo FNR is unknown at present, the embryo-specific expression of a NR gene in rapeseed (Fukuoka et al. 1996) suggests that rice embryo FNR is involved in nitrate assimilation in the tissue as in the case of root-specific enzyme in rice (Aoki and Ida 1994), maize (Ritchie et al. 1994) and pea (Bowsher and Knight 1996) roots. Another possible function of embryo FNR is to generate Fd_{red} required for fatty acid desaturation during seed maturation and/or germination.

CHAPTER III

STRUCTURAL AND PHYLOGENETIC ANALYSIS OF RICE FERREDOXIN-NADP$^+$ OXIDOREDUCTASE cDNAs

Photosynthetic FNR was first purified from spinach leaves (Shin et al. 1963) and subsequently isolated from a large number of higher plants, eukaryotic algae (Bookjans et al. 1979) and cyanobacteria (Susor et al 1966; Rowel et al 1981; Javier et al. 1988; Knaff 1996). The amino acid sequences of FNRs were determined for *Spirulina platensis* (Yao et al. 1984) and spinach (Karplus et al. 1984). Recently a number of the primary structures of FNRs have been deduced from the corresponding gene structure. I have compiled the sequence data available up to date and used them for structural and phylogenetic analysis of the enzyme from higher plants, green algae, cryptophyte (*Cyanophora paradoxa*) and cyanobacteria. More recently FNR cDNAs have been cloned from tobacco cultured cells and heterotrophic tissues of several higher plants including my own cDNA clonings of rice root and embryo FNRs as described in Chapter II.

The amino acid sequences of these FNRs are compared and analyzed for classification and evolutional relationships by the construction of a phylogenetic tree based on the amino acid sequence homology.

Materials and Methods

Computer analysis

Homology alignment and calculation of homology (%) of the primary structure of FNR were done using the Clustal W program (Tompson et al. 1994) and maximum homology (DNASIS-Mac v3.5), respectively. A phylogenetic tree was constructed using the Clustal W program and the TreeView PPC program based on the amino acid sequences of the mature proteins.

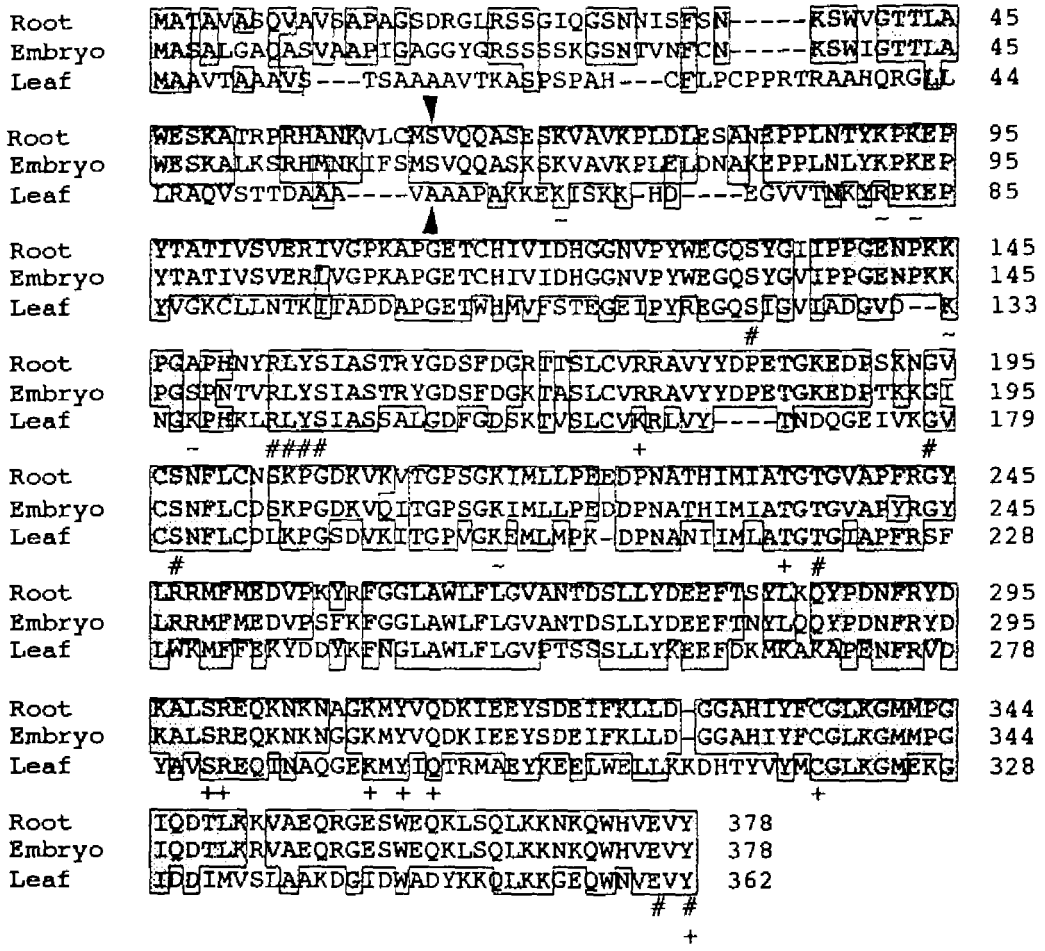


Figure 3-1. Comparison of the predicted amino acid sequences of FNR cDNAs from rice roots, embryos and leaves. The N-terminus of the mature protein of each FNR is indicated by arrowhead. Gray boxes show identical amino acid residues to rice root FNR protein. Dashes (–) indicate gaps introduced to maximize alignment. The location of FAD binding site (###), ferredoxin binding site (~~~), and $NADP^+$ binding site (+++) are marked under the alignment of the sequence. Assignment of the residues involved in the binding sites was followed to Karplus et al. (1991) and Jelesarov et al. (1993).

Results and Discussion

Comparison of the deduced amino acid sequences of rice root, embryo and leaf FNR is shown in Figure 3-1. Although there are less identical amino acids in the transit peptides (57%), comparison of the predicted amino acid sequences of the mature proteins revealed an extensive homology between rice root and embryo FNRs amounting to 90% identity. Rice embryo FNR

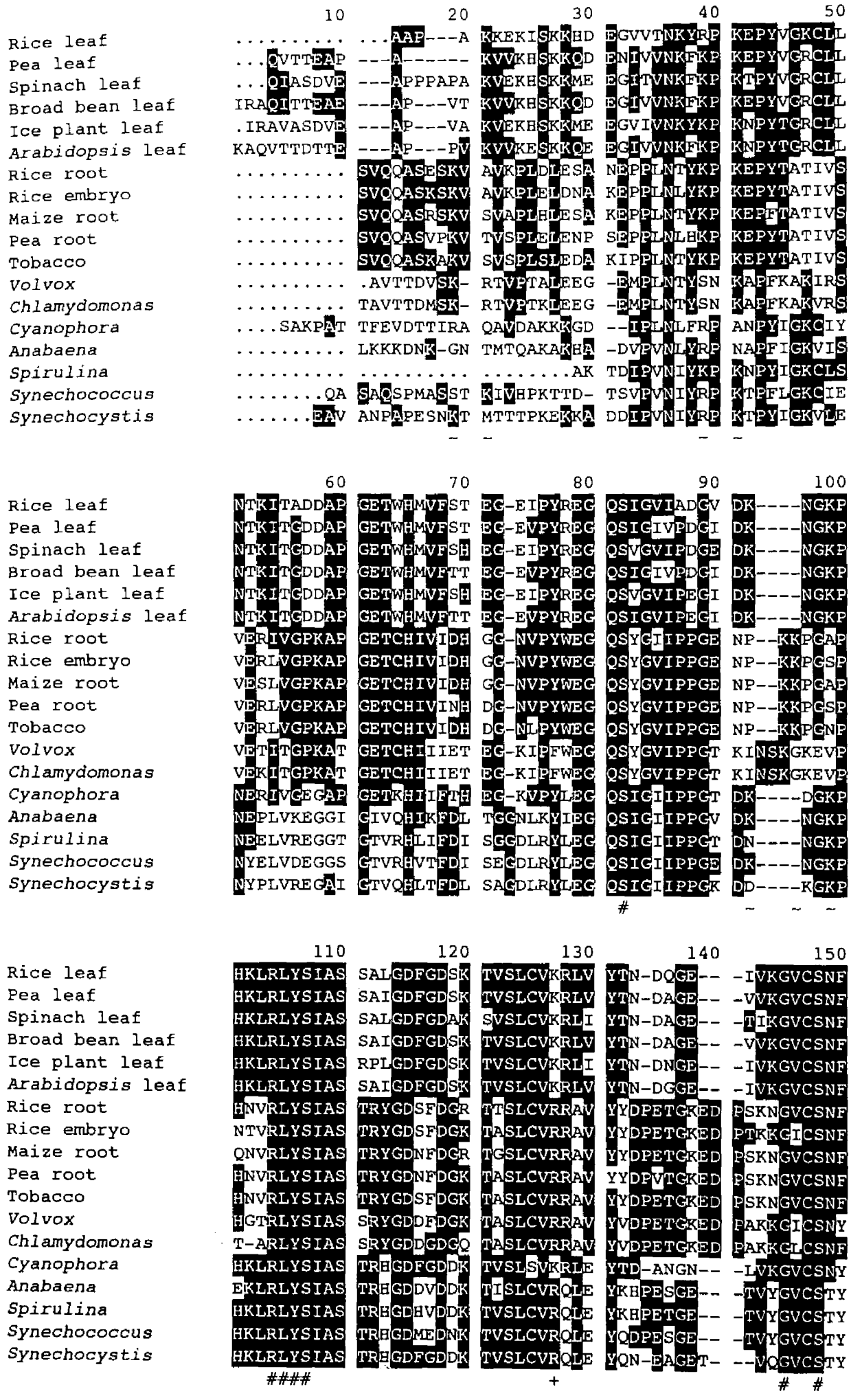
shows higher identities to the root enzyme than to the leaf protein, homology between the embryo and leaf enzyme is 49% in the mature protein and 28% in the transit peptide. In addition, homology between the leaf and the root enzyme is 49% in the mature protein and 28% in the transit peptide. Despite the lack of apparent homologies in the transit sequences, there are a number of highly conserved segments in the mature proteins from rice root, embryo and leaf FNRs. These identical amino acid residues have been indicated to be involved in the binding to FAD (100%), Fd (66.7%) and $NADP^+$ (87.5%) (Figure 3-1). Even if these residues differ between three FNRs, they are essentially the same, because they are analogous amino acids. Significant degree of similarity between rice embryo and root FNR is also recognized in the nucleotide sequence of the 3' untranslated regions (Aoki and Ida 1994; Aoki et al. 1996). These data are consistent with the presence of enzymatically and immunologically distinct FNR in photosynthetic and nonphotosynthetic tissues (Morigasaki et al. 1990b; 1990c).

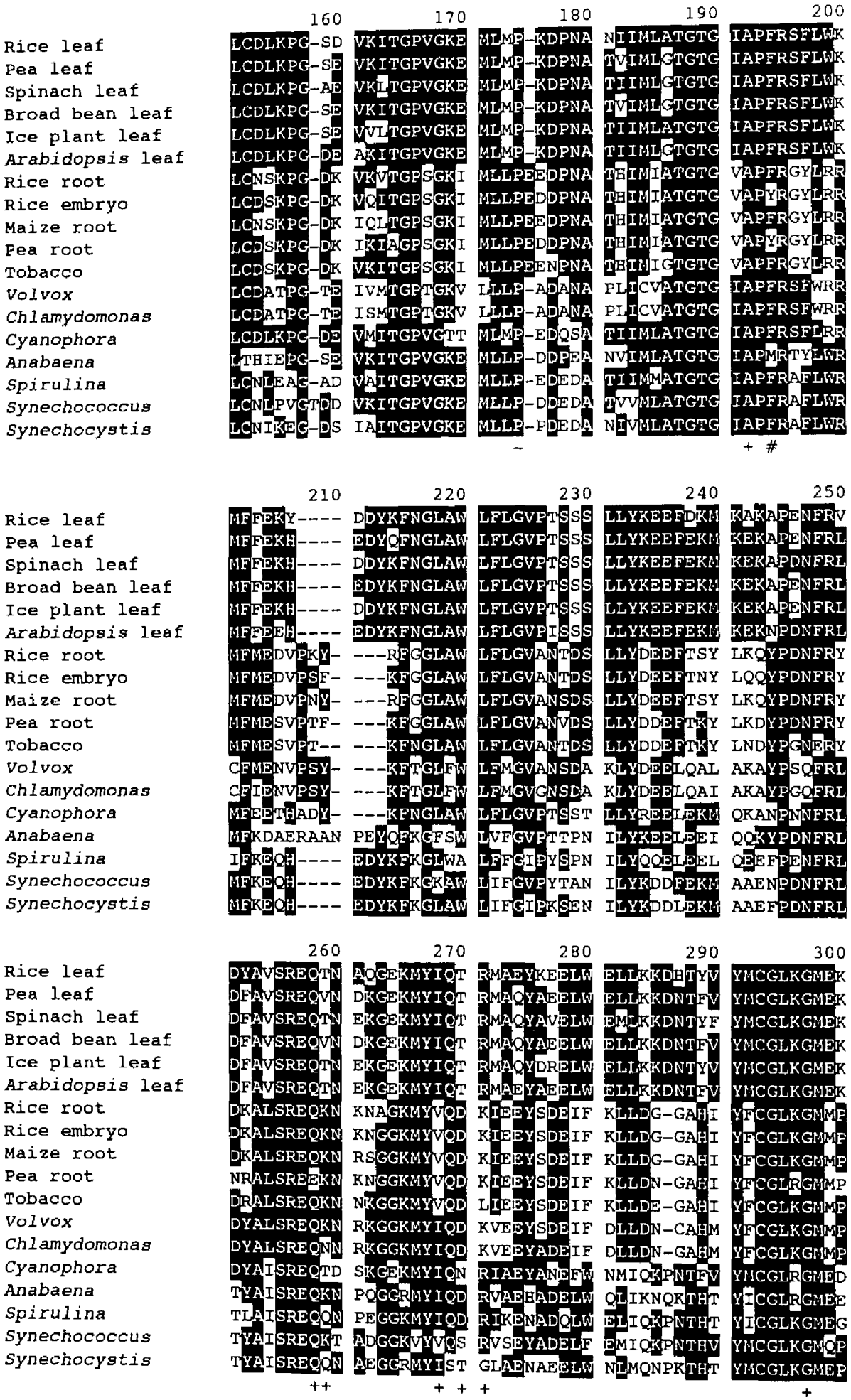
Sequence alignment of the mature proteins of FNRs from higher plants, cyanobacteria, green algae and *Cyanophora* is shown in Figure 3-2. The amino acid residues involved in the binding to FAD, Fd and $NADP^+$ are also highly conserved in FNRs whose sequence are available at present (Figure 3-2).

Homology (%) of FNR mature proteins from higher plants, cyanobacteria, green algae and *Cyanophora* is shown in Table 3-1. Rice leaf FNR are extremely homologous to the other photosynthetic enzyme in higher plants. For example, identity of rice leaf FNR to the enzyme from ice plant, pea, spinach, broad bean and *Arabidopsis thaliana* shows 85%, 84%, 81%, 82% and 82%, respectively (Table 3-1). Similarities found among the higher plant FNR sequences suggest that their structural genes are highly conserved irrespective of the dicots and monocots plants (Figure 3-2).

Although overall sequence identities show only 49 to 51% homology in the mature protein among rice root FNR and the other photosynthetic enzymes, comparison of the deduced protein sequence of rice root FNR with the other nonphotosynthetic enzymes revealed extensive homology in higher plants. The mature protein of rice root FNR has 88%, 92% and 85% identity with the tobacco cultured cells, maize root and pea root enzymes, respectively (Table 3-1).

Homology (%) of rice embryo FNR to the enzymes from higher plants, green algae, *Cyanophora* and cyanobacteria is shown in Table 3-1. Sequence homology is more conspicuous in the mature protein region between rice embryo and other FNRs from nonphotosynthetic tissues (88 to 90%) as compared with similarities to FNRs from the photosynthetic tissues (49 to 51%) (Table 3-1).


10 20 30 40 50
Rice leaf
Pea leaf
Spinach leaf
Broad bean leaf
Ice plant leaf
Arabidopsis leaf
Rice root
Rice embryo
Maize root
Pea root
Tobacco
Volvox
Chlamydomonas
Cyanophora
Anabaena
Spirulina
Synechococcus
Synechocystis
60 70 80 90 100
110 120 130 140 150


160 170 180 190 200
Rice leaf
Pea leaf
Spinach leaf
Broad bean leaf
Ice plant leaf
Arabidopsis leaf
Rice root
Rice embryo
Maize root
Pea root
Tobacco
Volvox
Chlamydomonas
Cyanophora
Anabaena
Spirulina
Synechococcus
Synechocystis
210 220 230 240 250
260 270 280 290 300

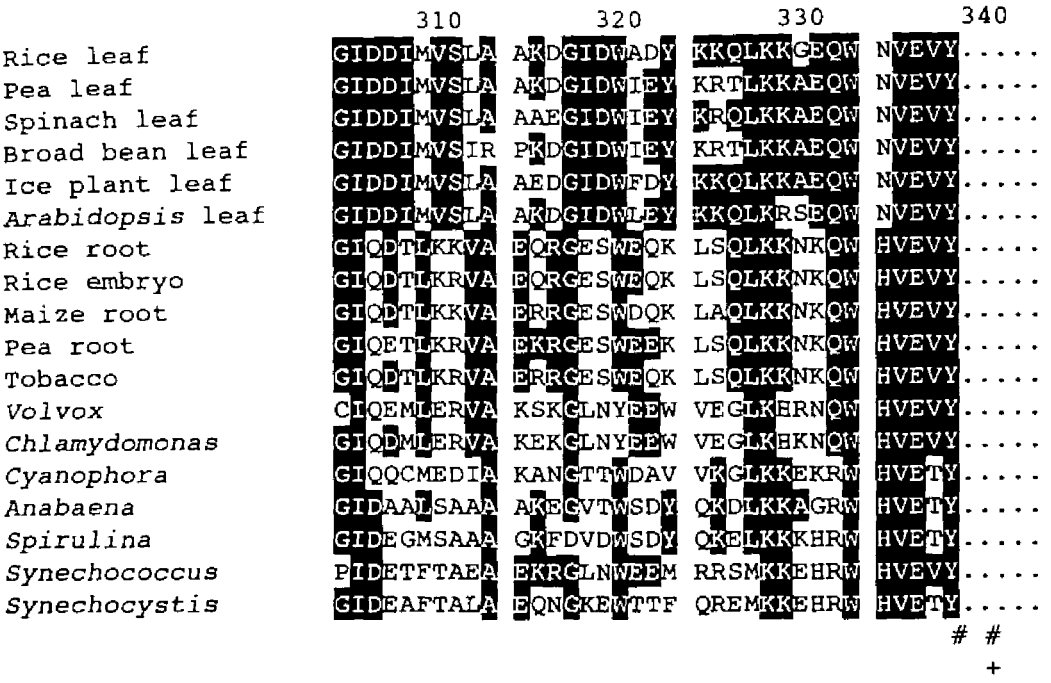


Figure 3-2. Comparison of the mature protein sequences of FNRs from higher plants, green algae, *Cyanophora* and cyanobacteria. Amino acid residues that are identical are blocked. Dashes (–) indicate gaps introduced to maximize alignment. The location of FAD binding site (###), ferredoxin binding site (---), and $NADP^+$ binding site (+++) are marked below the sequence alignmen. The proteins of broad bean, *Arabidopsis, Volvox, Chlamydomonas, Cyanophora, Anabaena, Synechococcus* and *Synechocystis* FNR have been truncated at the termini to mach the start of the spinach leaf FNR mature protein. The proteins of rice embryo, maize root, tobacco cultured cells and pea root FNR have been truncated at the termini to mach the start of the rice root FNR mature protein. Each row is the deduced amino acid sequence from rice leaf (*Oryza sativa*, Accession No. D17790, Aoki et al. 1994), pea leaf (*Pisum sativum*, Accession No. M21449, Newman and Gray 1988), spinach leaf (*Spinacia oleracea*, Accession No. X07981, Karplus et al. 1984; Jansen et al. 1988), broad bean leaf (*Vicia faba*, Accession No. U14956, Lax and Cary, unpublished), ice plant leaf (*Mesembryanthemum crystallinum*, Accession No. X13884, Michalowski et al. 1989), *Arabidopsis* leaf (*Arabidopsis thaliana*, Ida et al. unpublished), rice root (*Oryza sativa*, Accession No. D17410, Aoki and Ida 1994), rice embryo (*Oryza sativa*, Accession No. D87547, Aoki et al. 1996), maize root (*Zea mays*, Accession No. T18890, Ritchie et al. 1994), pea root (*Pisum sativum*, Accession No. X99419, Bowsher and Knight 1996), tobacco cultured cells (*Nicotiana tabacum*, Ida et al., unpublished), *Volvox* (*Volvox carteri*, Accession No. U22328, Choi et al., unpublished), *Chlamydomonas* (*Chlamydomonas reinhardtii*, Accession No. U10545, Kitayama et al. 1994), *Cyanophora* (*Cyanophora paradoxa*, Accession No. X66372, Jakowitsch et al. 1993), *Anabaena* (*Anabaena* sp. PCC 7119, Accession No. X72394, Fillat et al. 1990), *Spirulina* (*Spirulina* sp., Accession No. A00531, Yao et al. 1984), *Synecchococcus* (*Synechococcus* sp. PCC 7002, Accession No. J05366, Schluchter and Bryant 1992) and *Synechocystis* (*Synechocystis* sp., Accession No. X94297, Thor Van, unpublished).

Table 3-1. Sequence homology among FNRs from higher plants, green algae, *Cyanophora* and cyanobacteria.

	Number of residues	Rice leaf	Rice root	Rice embryo	Maize root	Tobacco chloroplasts	Pea root	Pea leaf	Spinach leaf	Ice plant leaf	Broad bean leaf	*Arabido psis* leaf	*C. reinhardtii*	*Volvox*	*C. paradoxa*	*Anabaena*	*Spirulina*	*Synecho coccus*
Rice leaf	307 (304)																	
Rice root	378 (316)	45 (49)																
Rice embryo	378 (316)**	44 (49)	85 (90)															
Maize root	327 (316)**	46 (49)	86 (92)	76 (88)														
Tobacco chloroplasts	375 (316)**	47 (50)	78 (83)	79 (88)	75 (86)													
Pea root	378 (316)**	45 (48)	77 (85)	78 (88)	75 (85)	80 (88)												
Pea leaf	360 (308)	75 (84)	48 (50)	46 (50)	45 (49)	47 (51)	46 (49)											
Spinach leaf	369 (314)	73 (81)	47 (50)	44 (50)	46 (50)	48 (51)	46 (49)	81 (86)										
Ice plant leaf	365 (313)	76 (85)	49 (52)	46 (51)	47 (51)	48 (51)	46 (49)	82 (85)	86 (89)									
Broad bean leaf	361 (314)*	74 (82)	47 (49)	45 (49)	45 (48)	47 (51)	46 (49)	96 (95)	80 (85)	80 (84)								
Arabido psis leaf	360 (314)*	76 (82)	48 (51)	46 (50)	47 (50)	49 (51)	48 (51)	83 (88)	78 (84)	80 (86)	83 (88)							
C. reinhardtii	354 (314)*	45 (48)	53 (60)	51 (61)	57 (62)	55 (60)	54 (59)	46 (49)	45 (48)	45 (49)	46 (48)	47 (50)						
Volvox	346 (314)*	45 (47)	56 (61)	56 (62)	59 (62)	55 (61)	56 (61)	46 (47)	44 (47)	45 (48)	46 (47)	47 (49)	88 (92)					
C. paradoxa	(314)*	56 (61)	47 (50)	47 (50)	46 (48)	46 (50)	46 (49)	58 (63)	56 (61)	58 (61)	58 (63)	58 (63)	45 (48)	47 (49)				
Anabaena	(314)*	(53)	(46)	(47)	(46)	(45)	(44)	(51)	(47)	(47)	(50)	(50)	(46)	(45)	(55)			
Spirulina	(294)	(53)	(47)	(47)	(46)	(46)	(45)	(54)	(52)	(54)	(52)	(54)	(45)	(45)	(57)	(66)		
Synecho coccus	(314)*	(55)	(49)	(48)	(48)	(48)	(49)	(54)	(54)	(54)	(53)	(52)	(46)	(46)	(56)	(58)	(66)	
Synecho cystis	(314)*	(55)	(46)	(45)	(45)	(46)	(45)	(54)	(53)	(53)	(52)	(55)	(44)	(43)	(57)	(62)	(68)	(71)

Figure in the first column indicates number of the amino acid residues of the precursor protein and figure in parenthesis represents number of the amino acid residues of the mature protein for the FNR. Sequence homology (%) in each pair was calculated by a maximum matching program (DNASIS -Mac v3.5). The proteins marked with an asterisk (*) and two asterisks (**) have been truncated at the termini to mach the start of the spinach leaf and rice root FNR mature protein, respectively. Sources of the protein sequence are described in Figure 3-2.

Cyanobacterial FNRs has low degrees of homology (lower than 55%) to photosynthetic FNRs of higher plants as well as their nonphotosynthetic enzymes. Green algal FNRs show somewhat higher sequence identity to the nonphotosynthetic enzymes (59 to 62%) than to the photosynthetic enzymes (47 to 50%). *Cyanophora* FNR resembles photosynthetic FNRs (61 to 63%) rather than to the nonphotosynthetic enzymes (48 to 50%) (Table 3-1).

Figure 3-3 shows a phylogenetic tree of FNRs based on the sequence homology of the FNR mature proteins from higher plants, green algae, *Cyanophora* and cyanobacteria. Sequence similarities can be divided into four separate groups, that is, FNRs from photosynthetic tissue of higher plants (group I), nonphotosynthetic tissue of higher plants (group II), green algae (group III) and cyanobacteria (group IV) with the exception of the *Cyanophora* enzyme. Within group I and group II, sequence homology is higher than 80%, whereas similarities between group I and group II are lower than 50%. It is interesting to know that green algal FNRs are closer to nonphotosynthetic type FNRs of higher plants (group II) than to the photosynthetic type counterpart of higher plants (group I), although green algae carry out the oxygenic photosynthesis similar to higher plants. The phylogenetic tree supports the observation that the divergence of photosynthetic type and nonphotosynthetic type FNRs started even before the divergence of the monocots and the dicots of higher plants.

Phylogenetic analysis suggests that cyanobacterial FNR appeared at first in the course of evolution and then divided into the photosynthetic and nonphotosynthetic enzymes independently at the time of the emergence of higher plants.

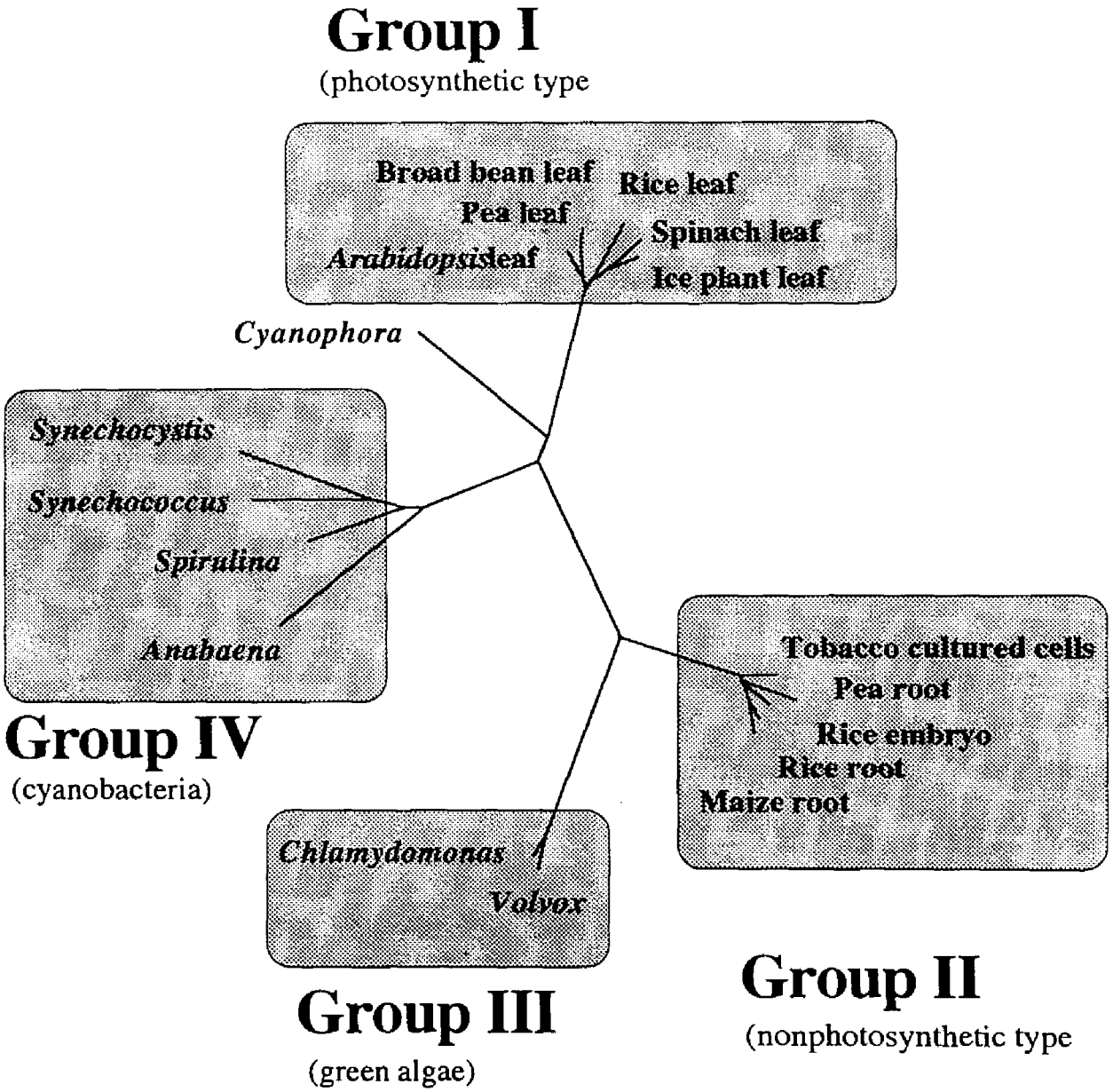


Figure 3-3. Phylogenetic analysis of FNR based on the amino acid sequences of the mature proteins. Alignmenst of the sequences and determination of pairwise genetic distance were created using the Clustal W program and the construction of the phylogenetic tree was made using TreeView PPC program. Sources of the sequence data are given in Figure 3-2.

CHAPTER IV

ESTIMATION OF COPY NUMBER OF THE RICE FERREDOXIN-NADP$^+$ OXIDOREDUCTASE GENES

As described in Chapter II, it was revealed that three FNR cDNAs are expressed in different tissues or organs in rice, indicating that rice carries at least three FNR genes in the haploid genome. Although copy number of FNR genes has been estimated in some higher plants, a single species of FNR cDNA was used as a probe in these experiments which resulted in the estimation of a single copy gene for spinach leaf FNR (Newman and Gray 1988) and one or two copy genes for pea leaf (Oelmüller et al. 1993) and maize root FNR (Ritchie et al. 1994).

Preliminary work indicated that the rice leaf FNR cDNA does not hybridize to the rice root FNR cDNA, vice versa, under high stringency conditions. These findings suggested that a single species of DNA probes does not reveal whole set of FNR gene copies in a given plant species. Differential genomic Southern analysis was conducted to ascertain the complexity of the FNR gene family in rice with the use of the rice leaf, root or embryo FNR cDNA as a DNA probe. The results indicate that there is at least one copy of the FNR genes corresponding to each FNR cDNA in rice.

Materials and Methods

Isolation of nuclear DNA

Nuclear DNA was isolated from rice leaves according to Sugiura (1989). Five g of fresh leaves were chilled with liquid nitrogen and pulverized to a fine powder. The pulverized tissue was mixed gently with 5 ml of 2 × CTAB solution (2% CTAB, 100 mM Tris-HCl (pH 8.0), 1.4 M sodium chloride and 1% PVP) at 70 °C to wet thoroughly and incubated for 10 min at 55 °C. Then the homogenate was extracted with an equal volume of chloroform/ isoamyl alcohol (24:1), mixed gently for 30 min, centrifuged for 15 min at 2,800 rpm and recovered the top (aqueous) phase. This extraction was repeated once more with 1 × CTAB solution. The recovered aqueous phase (10 ml) was mixed with a 1/10 volume (1 ml) of 10% CTAB solution (10% CTAB and 700 mM sodium chloride) and an equal volume (11 ml) of precipitation buffer

(1% CTAB, 50 mM Tris-HCl (pH 8.0) and 10 mM EDTA), mixed well for 30 min by inversion, centrifuged for 15 min at 2,800 rpm and removed the supernatant. The pellet was resuspend in 5 ml NaCl-TE solution (1 M sodium chloride, 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA) at 55 °C and nuclear DNA was reprecipitated by adding an equal volume (5 ml) of 2-propanol and was centrifuged for 10 min at 2,800 rpm. The pellet was washed with 70% ethanol, dried and resuspended in 500 μl pure water. Two mg of genomic DNA was prepared from 5 g of rice leaves.

Genomic Southern hybridization

Rice nuclear DNA (100 μg) was digested overnight with 200 units each of *Bam* HI, *Eco* RI, *Pst* I, *Sal* I and *Xho* I in a final volume of 100 μl. These digested nuclear DNAs were subjected to electrophoresis on a 1.2% agarose gel. The gel was soaked in depurination solution (250 mM hydrochloric acid) for 10 min, denaturation solution (500 mM sodium hydroxide and 1.5 M sodium chloride) for 30 min and neutralization solution (500 mM Tris-HCl (pH 7.4) and 1.5 M sodium chloride) for 30 min. Nuclear DNAs were transferred to nylon membrane (Hybond-N+, Amersham) with 20 × SSC (3 M sodium chloride and 300 mM sodium citrate). The DNA fragment between *Sal* I and *Eco* RI site of the rice leaf FNR cDNA (L9) was used as a leaf FNR probe, fragment between *Pst* I and *Eco* RI site of the rice root FNR cDNA (R14) was used as a root FNR probe and fragment between *Pst* I and *Bam* HI site of the rice embryo FNR cDNA (E1) was used as an embryo FNR probe. Hundred ng of each fragment was labeled with ECL direct nucleic acid labeling and detection systems (Amersham). The membrane was rinsed in 50 ml of 2 × SSC and prehybridised in 10 ml of ECL gold hybridization buffer with 5% w/v blocking agent and 500 mM NaCl for 1 h at 42 °C. Then labeled cDNA was added to the membrane and incubated for 16 to 20 h. The hybridized membrane was washed with 50 ml of 0.2 × SSC and 0.4% SDS three times for 10 min at 55 °C and twice for 5 min of with 2 × SSC. The signal was generated with 2 ml of detection solution I and II mixture for 5 min, removed the solution and finally exposed to an X-ray film.

Rehybridization

Rehybridization methods were done according to Sakai (1991). After detection, the probes were removed to wash twice in 50 ml of 100 mM sodium hydroxide for 1 h, rinsed twice in 50 ml of 2 × SSC and prehybridized again as described above.

Results and Discussion

When the leaf FNR probe was used, a single band of about 30, 3.3, 21 and 16 kb was observed in the *Bam* HI, *Eco* RI, *Pst* I and *Sal* I digested DNA, respectively. But in the *Xho* I digested DNA, there was a weakly hybridizing band of about 4.7 kb in addition to the strong band of about 30 kb (Figure 4-1, A). There are no *Bam* HI, *Eco* RI, *Pst* I, *Sal* I and *Xho* I sites in the *Sal* I-*Eco* RI fragment of the leaf FNR cDNA used as a probe. Intensity of the bands appeared in the *Xho* I digest suggests that the leaf FNR gene has a single copy gene in rice. However, because of the possible presence of introns in the nuclear genes, the isolation of genomic clones will be necessary to substantiate this suggestion. Copy number of the leaf FNR gene has been estimated for pea (Newman and Gray 1988) and spinach (Oelmüller et al. 1993) using their corresponding cDNAs as probes, respectively. These results indicated that only a single band was observed in each digest, suggesting that the leaf FNR cDNA does not hybridize to the nonphotosynthetic FNR genes. These experiments also suggest that there are a single copy gene for spinach leaf FNR and one or a few copy genes for pea leaf FNR.

On the other hand, when the root or embryo FNR probe was used, more than one band appeared in each digest. When the root FNR cDNA was used, there were three bands in the *Bam* HI digest (16, 4.0 and 2.7 kb), two bands in the *Eco* RI digest (5.1 and 2.1 kb), three bands in the *Pst* I digest (5.1, 3.6 and 1.9 kb), a single band in the *Sal* I digest (28 kb) and a single band in the *Xho* I digest (25 kb) (Figure 4-1, B).

With the use of the embryo FNR probe, there were three bands in the *Bam* HI digest (16, 4.0 and 2.7 kb), two bands in the *Eco* RI digest (5.1 and 2.1 kb), three bands in the *Pst* I digest (5.1, 3.6 and 1.9 kb), a single band in the *Sal* I digest (30 kb) and two bands in the *Xho* I digest (20 and 17 kb) (Figure 4-1, C).

The results indicate that most of the positions of several bands are identical in these digests with both root and embryo FNR probes. The overlapped positions are as follows: 16, 4.0 and 2.7 kb in the *Bam* HI digest; 5.1 and 2.1 kb in the *Eco* RI digest and 5.1, 3.6 and 1.9 kb in the *Pst* I digest. The results suggest that the bands observed at the same positions with these probes are originated in the same genes.

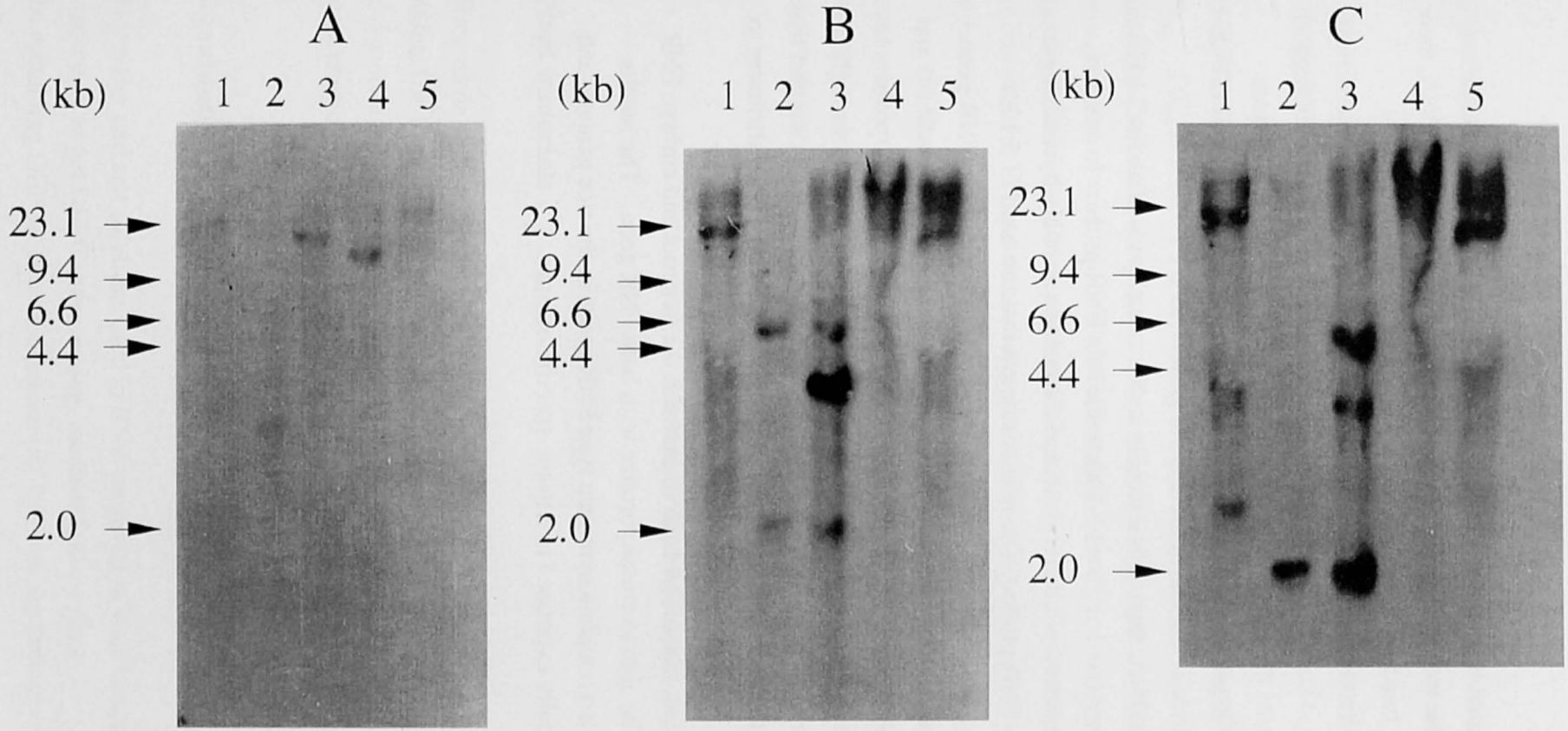


Figure 4-1. Determination of the FNR gene copy number in the rice genome. DNA blots were hybridized with the rice leaf (A), root (B) and embryo FNR cDNA clone. Lane1, *Bam* HI; lane2, *Eco* RI; lane3, *Pst* I; lane4, *Sal* I and lane5, *Xho* I.

Further analysis indicated that intensity of the signals which appeared at the same positions was different with the root and embryo probes. In the *Bam* HI digested DNA, there were two weakly hybridizing bands of about 4.0 and 2.7 kb and there was a strongly hybridizing band of about 16 kb to the root FNR probe. In contrast, there were three strongly hybridizing band of about 16, 4.0 and 2.7 kb to the embryo FNR probe. The results suggest that the band of 16 kb represents the root FNR gene and the bands of 4.0 and 2.7 kb are ascribed to for the embryo FNR gene. Appearance of two bands to the embryo FNR cDNA probe may be due to the presence of introns in the nuclear genes.

In the *Eco* RI digested DNA, there was a weakly hybridizing bands of about 2.1 kb and there was a strongly hybridizing band of about 5.1 kb to the root FNR probe. In contrast, there was a weakly hybridizing band of about 5.1 kb and there was a strongly hybridizing band of about 2.1 kb to the embryo FNR probe. The results suggest that the band of 5.1 kb represents the root FNR gene and the band of 2.1 kb corresponds to the embryo FNR gene.

In the *Pst* I digested DNA, there were two weakly hybridizing bands of about 5.1 and 1.9 kb and there was a strongly hybridizing band of about 3.6 kb to the root FNR probe. In contrast, there was a weakly hybridizing band of about 3.6 kb and there were two strongly hybridizing band of about 5.1 and 1.9 kb to the embryo FNR probe. The results suggest that the band of 3.6 kb represents the root FNR gene and the bands of 5.1 and 1.9 kb attributes to the embryo FNR gene.

Since there are significant homologies (76%) between the rice root and embryo FNR cDNA, each FNR probe may be able to crosshybridize with both FNR gene. The results suggest that there are at least two nonphotosynthetic type FNR genes in the rice genome and these FNR genes are differentially expressed in a tissue-specific manner.

CHAPTER V

INDUCTION OF THE RICE ROOT AND LEAF FERREDOXIN-$NADP^+$ OXIDOREDUCTASE mRNA

FNR in nonphotosynthetic tissues is thought to catalyze the electron transfer from NADPH to Fd_{ox}. Fd_{red} is used for Fd-dependent enzymes such as NiR and GOGAT which are involved in the nitrate assimilation systems. Recently, Bowsher et al. (1993) demonstrated that a protein immunoreactive with anti-leaf FNR increases upon nitrate exposure to pea roots. A four-fold increase in the NADPH-dependent FNR activity was observed with an increase in the amount of this protein. These results suggest that FNR in nonphotosynthetic tissues is induced by nitrate.

In Chapter II, I have isolated FNR cDNAs from nonphotosynthetic tissue (rice roots and embryos) as well as photosynthetic tissues (rice leaves). I have demonstrated that the rice root FNR mRNA is induced rapidly and transiently by nitrate. I also demonstrated that the rice leaf FNR mRNA is induced by light.

Materials and Methods

Plant materials

Rice seeds were soaked overnight in water and grown hydroponically on 0.1 mM calcium sulfate for 10 days at 28 °C in the dark. Seedlings were treated with 7 mM potassium nitrate and roots were harvested, or treated by light and leaves were harvested. Roots and leaves were placed into liquid nitrogen and stored at -80 °C.

Denatuation of ribonuclease

Any water and salt solutions used in RNA preparation were treated with 0.2% DEPC, shaked vigorously to get DEPC into solution, incubated overnight at 37 °C and autoclaved to inactivate the remaining DEPC. Solution containing Tris was not treated with DEPC, because Tris reacts

with DEPC to inactivate it. Glassware was baked at 200 °C for more than 3 h. Plastic ware was soaked in 5% hydrogen peroxide for more than 2 h and autoclaved or dried.

Total RNA preparation

Preparation of total RNA were carried out according to Matsui et al. (1990). One g of fresh rice roots or leaves were rapidly chilled with liquid nitrogen and homogenized in 4 ml of denaturing solution (50% guanidine thiocyanate and 25 mM Tri-sodium citrate dehydrate), 28 μl of 2-ME and 400 μl of 20% sodium lauryl sarcosinate. The pulverized tissues were transferred to a 15 ml polypropylene tube (Corning), vortexed for 2 min, added with 500 μl of water-saturated phenol and 500 μl of chloroform, vortexed for 1 min and centrifuged for 10 min at 6,000 rpm at 4 °C. The top (aqueous) phase was recovered in a new polypropylene tube and the aqueous phase was treated again with phenol/ chloroform. The aqueous phase was precipitated with 10% volume of 3 M sodium acetate (pH 5.5) and 250% volume of ethanol and finally centrifuged for 10 min at 2,800 rpm at 4 °C. RNA pellet was dissolved in 600 μl of TE, transferred to microtube and extracted with water-saturated phenol twice and with chloroform once more. The supernatant was blended with an equal volume of 4 M lithium chloride anhydride, mixed thoroughly and incubated overnight at 4 °C. The mixture was centrifuged for 30 min at 15,000 rpm at 4 °C. The pellet was washed with 70% ethanol, dried and resuspended in 100 μl of water. The solution was extracted with chloroform once more and precipitated with ethanol again. The pellet was washed with 70% ethanol, dried and resuspended in 20 μl of water.

Northern hybridization

Four μg of total RNA were dot-blotted to a nylon membrane (Hybond-N+, Amersham). The membrane was dried, rinsed in 10 ml of 2 × SSC and baked for 2 h at 80 °C. The membrane was prehybridized in 5 ml of ECL gold hybridization buffer with 5% w/v blocking agent and 500 mM sodium chloride for 1 h at 42 °C. The full-length cDNA clones from rice roots (R14) and leaves (L9) were used as probes. Each probe was labeled and detected with ECL direct nucleic acid labeling and detection systems (Amersham). The hybridization and detection were done as described in Chapter IV. Autoradiograms were scanned with a Shimadzu CS-9000 dual-wavelength flying-spot scanning densitometer.

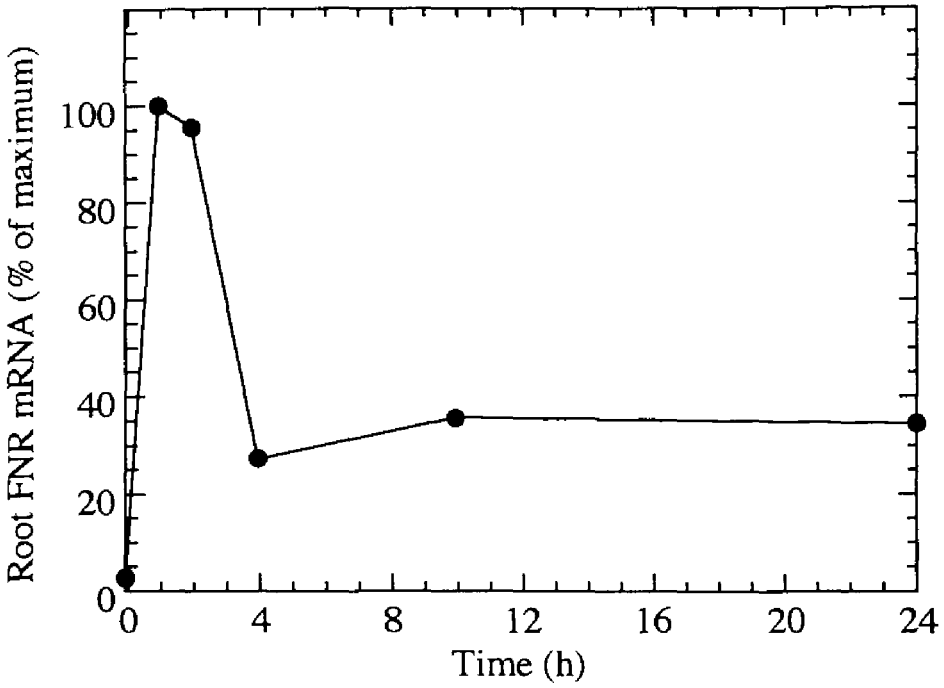


Figure 5-1. FNR mRNA accumulation after nitrate induction in rice roots. FNR mRNA was measured by dot blots hybridized with the *Eco* RI fragment from clone R14 as described in text.

Results and Discussion

Rice roots were harvested at 0, 1, 2, 4, 10 and 24 h after nitrate treatment. The mRNA accumulation for rice root FNR was examined with the use of the root FNR cDNA as a probe. There was a very low level of the FNR mRNA in the roots grown on N-free solution. Addition of nitrate to rice seedlings induced a rapid accumulation of the transcript. The induction reached its maximum level in 1 to 2 h after the addition of nitrate (increased over 34-fold) and decreased rapidly thereafter (Figure 5-1). Maximum level of the transcript was reached earlier than that in the leaf tissue and any significant increase in the FNR mRNA was not observed when ammonium nitrogen was added as a nitrogen source (unpublished data). The general pattern of the accumulation and decline of the FNR transcript is very similar to those reported for NR (Melzer et al. 1989) and NiR (Kramer et al. 1989) in barley roots, GS and GOGAT in maize roots (Redinbaugh and Campbell 1993). Recently, Fd type protein and FNR have been described to be induced in pea root plastids during nitrate assimilation (Bowsher et al. 1993). And after completion of this work (Aoki and Ida 1994), Ritchie et al. (1994) reported that the FNR mRNA in maize roots was accumulated specifically in response to nitrate, since neither potassium nor ammonium treatment of roots caused the transcript

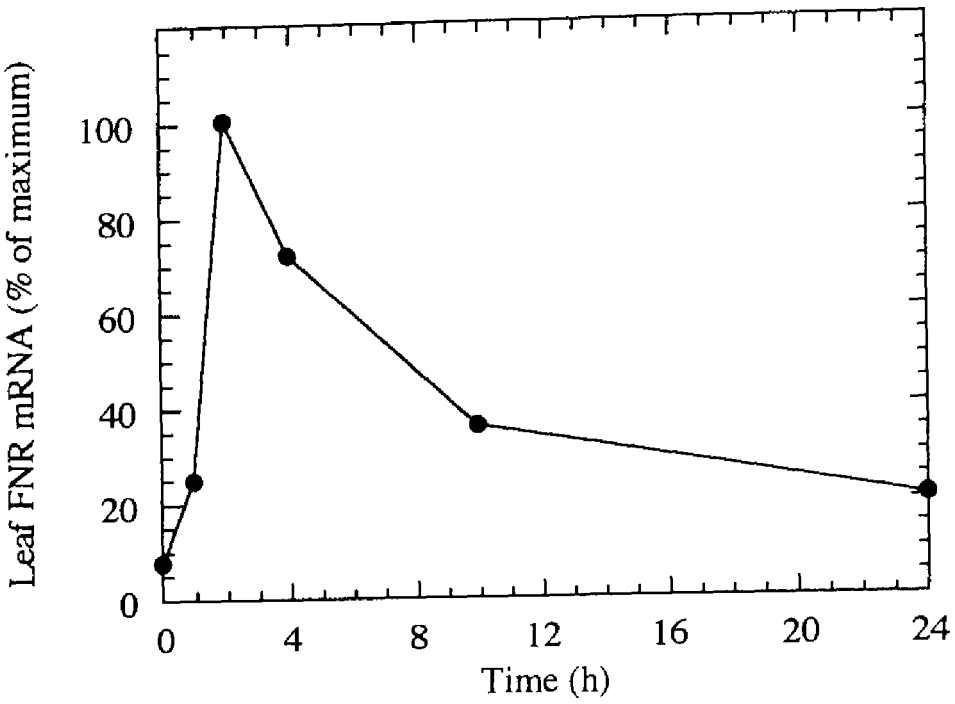


Figure 5-2. FNR mRNA accumulation after light induction in rice leaves. FNR mRNA was measured by dot blots hybridized with the *Eco* RI fragment from clone L9 as described in text.

accumulation. These results demonstrate that the FNR transcript is induced by nitrate in rice and maize roots. Furthermore, close similarities of the general induction patterns of the FNR transcript to those of NR and NiR in root tissues suggests the concurrent induction of the nitrate assimilatory enzymes with nitrate.

The mRNA expression for rice leaf FNR was examined with the use of the rice leaf FNR cDNA as a probe. There was a low level of the leaf FNR mRNA in 10-day old etiolated leaves grown on 0.1 mM calcium sulfate. The FNR mRNA transcript was also induced rapidly by exposure of light to seedlings. The induction reached its maximum level in 2 h after the irradiation of light and decreased gradually thereafter (Figure 5-2). The delay of induction compared to the root FNR mRNA might be caused by the time lag of chloroplast formation. But the increase in the leaf FNR transcript was lower (about 14-fold) than that of the root FNR mRNA. Continuous illumination has been reported to be necessary for the maintenance of high levels of FNR development in bean leaves (Haslett and Cammack 1976). Such light dependency of the leaf enzyme suggests that the regulatory systems are different between photosynthetic and nonphotosynthetic tissues. Perhaps the rice leaf FNR expression is regulated by the same way of the other photosynthetic enzymes in chloroplasts.

CHAPTER VI

THE GENOMIC ORGANIZATION OF THE RICE ROOT FERREDOXIN-NADP$^+$ OXIDOREDUCTASE

As described in Chapter II, three structurally distinct rice FNR cDNAs were isolated from the cDNA libraries from different organs or tissues. The deduced amino acid sequences indicated that a FNR cDNA found in the leaves corresponds to the photosynthetic FNR and the other two FNR cDNAs found in the roots and in the embryos correspond to the nonphotosynthetic enzymes. The latter two FNR cDNAs have much less similarities in the deduced amino acid sequences to photosynthetic FNR cDNAs of rice and other higher plant leaves. Expression of the photosynthetic FNR gene has been shown to be under phytochrome control (Oelmüller et al. 1993; Lübberstedt et al. 1994; Bowler and Chua 1994; Aoki et al. 1994). In contrast to the leaf gene, the root FNR gene is nitrate-inducible along with the genes involved in the nitrate assimilation pathway in the root tissues of higher plants (Melzer et al. 1989; Kramer et al. 1989; Redinbaugh and Campbell 1993; Aoki and Ida 1994).

Although a genomic clone encoding the spinach leaf FNR has been described which comprises the promoter and partial coding regions of the gene (Oelmüller et al. 1993), there is no report on the genomic structure of a gene whose expression is induced by nitrate in the root of higher plants. So, I undertook cloning and sequencing of the genomic clone corresponding to the rice root FNR cDNA.

Materials and Methods

Bacterial Strains and Plasmids

E. coli strain P2392 was used for screening of recombinant DNA manipulations. All cloning and sequencing procedures were performed with the phagemid vector pBS SK+.

Screening of the genomic library

A rice (*Oryza sativa* L. cv. Nihonbare) lambda EMBL3 genomic library constructed from genomic DNA partially digested to about 10 kb fragments with *Sau* 3AI was a gift from Professor Dr. Kunisuke Tanaka, Kyoto Prefectural University.

The genomic library was screened using the rice root FNR cDNA clone (R14) as a probe. The probe was labeled and detected with ECL direct nucleic acid labeling and detection systems (Amersham) as described in Chapter II. The primary washing solution used for the first screening was 0.2 × SSC containing 0.4% SDS. After the first screening, 10 positive plaques were obtained from 4×10^5 phage. In the second and the third screening, the primary washing solution was changed to 0.1 × SSC containing 0.4% SDS for high stringency. After the third screening, 5 positive plaques were obtained which were named GRFNR 5 to 9. Each positive clone was digested with several restriction enzymes, subjected to electrophoresis on a 1.2% agarose gel, transferred to a nylon membrane (Hybond N+, Amersham) and hybridized to the probe. All of the 5 clones contained the root FNR gene.

Clone GRFNR5 was digested with *Eco* RI and *Sac* I and the 1.6 kb insert was subcloned into pBS SK+ with *Eco* RI and *Sac* I site according to Hayashi et al. (1986) using a Takara DNA ligation kit and transformed into MV1184. Clone GRFNR5 was also digested with *Eco* RI and *Kpn* I and the 2.3 kb insert was subcloned into pBS SK+ with *Eco* RI and *Kpn* I site and transformed into *E. coli* MV1184. In addition, both inserts were digested with several restriction enzymes and short inserts were recloned into pBS SK+. Deletion subclones were generated using a Takara kilo-sequence deletion kit (Henikoff 1984). All subclones were sequenced for both directions using an Applied Biosystems sequencing kit with *Taq* DNA polymerase and -21M13, M13RP primers based on the dideoxy chain termination method (Sanger et al. 1977).

Primer extension analysis

Total RNA was isolated from rice roots induced with 10 mM potassium nitrate for 1 h as described in Chapter V. An oligonucleotide primer (5'-ATGGCCGATCCTGAGGGAAA-3') from positions +47 to +66 from translational start point Met of the rice root FNR gene was purchased from Krabou Co., Ltd. The primer was labeled the 5' end with biotin. Double-stranded DNA sequence analysis were performed by the dideoxy chain termination with *Tth* DNA polymerase and the oligonucleotide primer using a Toyobo sequence high cycle kit.

The 5' end of the rice root FNR mRNA was mapped using the primer extension protocol according to Triezenberg (1992). Fifty μg of total RNA, 10 μl of hybridization buffer (100 mM Tris-HCl (pH 8.3), 1.5 M potassium chloride and 10 mM EDTA), 4 μl of 6.6 ng/μl oligonucleotide primers and DEPC-treated H_2O were mixed to a final volume 100 μl in a microcentrifuge tube. The tube was sealed securely and submerged for 90 min at 65 °C and allowed to cool slowly to room temperature for 90 min. The total RNA was precipitated with ethanol, linsed with 70% ethanol and mixed with 2 μl of 5 × first strand buffer (250 mM Tris-HCl (pH 8.3), 375 mM potassium chloride, 15 mM magnesium chloride), 1 μl of 100 mM DTT, 1 μl of 10 × dNTP (2.5 mM each), 5.8 μl of DEPC-treated H_2O and 0.2 μl of 200U/μl superscript™ II reverse transcriptase (Gibco BRL). The mixture was incubated for 1 h at 42 °C and denatured the reverse transcriptase for 10 min at 90 °C. RNA degradation was performed by adding 0.2 μl of 500 mM EDTA and RNase solution (20 μg/ml DNase free-RNase A, 100 μg/ml salmon sperm DNA, 100 mM sodium chloride) for 1 h at 37 °C. Finally, 5 μl of stop solution of a Toyobo sequence high cycle kit was added to the mixture and 5 μl of the mixture was applied to electrophoresis. The products were analyzed on a 6% denaturing (8 M urea) acrylamide sequencing gel and visualized with a chemilunescent detection kit (Toyobo).

Results and Discussion

The rice root FNR gene was isolated by homologous hybridization with the rice root FNR cDNA. Five genomic clones named GRFNR 5 to 9 were isolated from 4 × 10^5 phage of the lambda EMBL3 genomic library from rice. Southern hybridization analysis and restriction maps showed all the clones to be identical. These results and genomic Southern analysis in Chapter IV are consistent with the finding that only a single root FNR gene copy is present per haploid genome of rice. A representative clone (GRFNR5) was chosen to digest with *Sal* I, which gave a 15 kb fragment. Further digestion of the DNA with *Sac* I, *Eco* RI and *Kpn* I yielded two fragments of 1.6 kb and 2.3 kb that comprised an entire region of the gene. The restriction map of the GRFNR5 clone is shown in Figure 6-1.

Nucleotide and the deduced amino acid sequences of the rice root FNR gene are shown in Figure 6-2. The exon/intron boundary follows the AG/GT rule of splice junction (Breathnach and Chambon 1981). The root FNR coding region consists of 6 exons (+1/+88, +186/+242, +558/+893, +1246/+1465, +1849/+2089 and +2307/+2775) interrupted by 5 introns (+89/+185, +243/+557, +894/+1245, +1466/+1848 and +2090/+2306). The protein

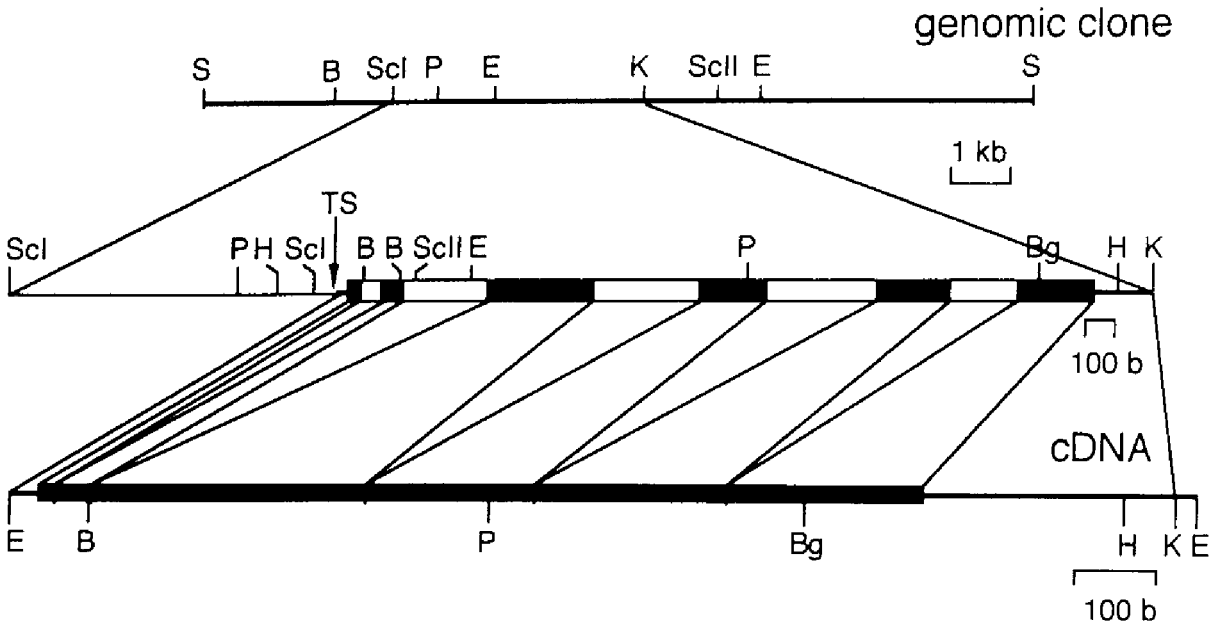


Figure 6-1. Restriction map of the root ferredoxin-NADP+ oxidoreductase gene of rice. The wide bar represents the protein coding regions, the open bar introns, the medium bar transcribed regions and the narrow bar untranscribed regions. The transcription start site (TS) is marked with a vertical arrow. B, *Bam* HI; Bg, *Bgl* II; E, *Eco* RI; H, *Hin* dIII; K, *Kpn* I; P, *Pst* I; S, *Sal* I; Sc I, *Sac* I and Sc II, *Sac* II.

coding sequence is completely identical with that from the rice root FNR cDNA. The 1st exon contains ATG translation start codon. The transit peptide stretches from the 1st to 3rd exon and the mature protein is encoded over the 3rd, 4th, 5th and 6th exon. The exons encoding only the transit peptide is much shorter (88 and 57 bp) than the remaining exons (336, 220, 241 and 259 bp). The binding site for Fd is in the 3rd, 4th and 5th exon and FAD binding site stretches over from the 3rd to the 6th exons. The $NADP^+$ binding site is located in the 4th, 5th and 6th exon and termination codon is found in the last coding segment (See Figure 3-1, for an assignment of the functional residues of FNR).

The transcription start site (TS) was determined by primer extension analysis with a synthetic primer complementary to the root FNR gene. The primer extension product was compared to the sequence ladders to determine its length. The reverse transcription yielded one major extension product of 66 bases (Figure 6-3), indicating that the TS was a nucleotide A that locates 64 bp upstream from the methionine initiation codon (Figure 6-2).

Figure 6-2. Nucleotide sequence of the rice root ferredoxin-NADP+ oxidoreductase gene. Sequence in bold capital letters indicate the exons and lower case letters represent the 5' and 3' flanking and intron sequences. Numbering of nucleotides begins at the transcription start site (designated +1). The TATA, CAAT and GC motifs are boxed. TGTAA sequences are double-underlined.

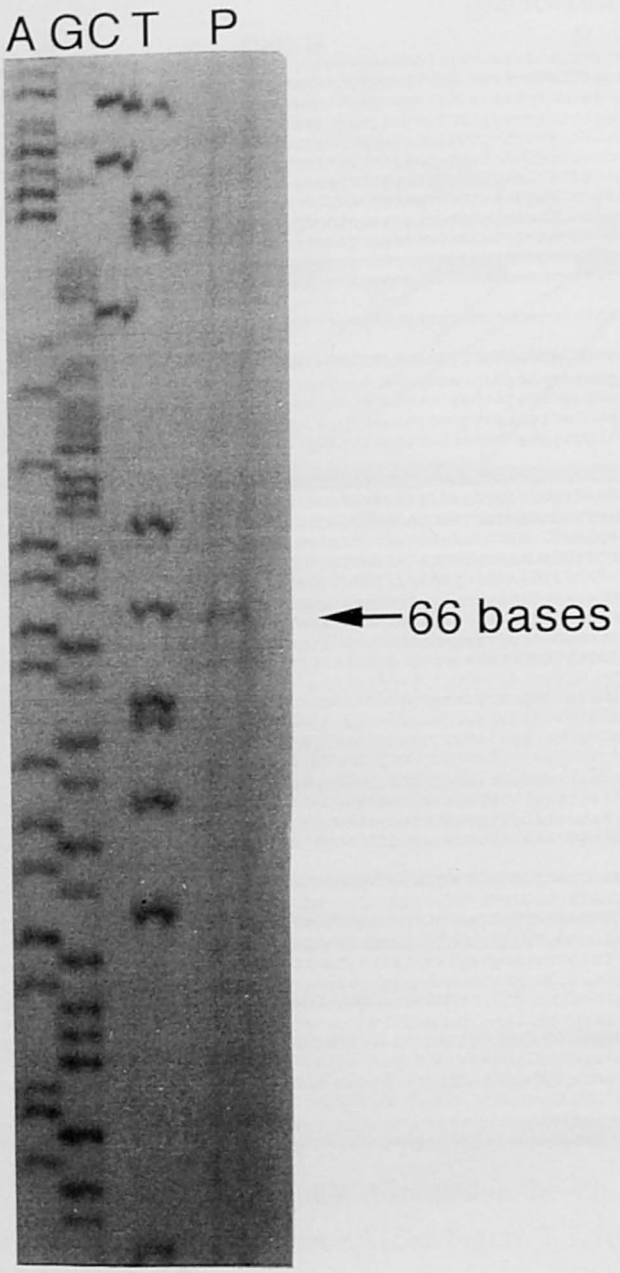


Figure 6-3. Determination of the transcription start site by primer extension analysis. Size of the nucleotide product is shown in the right of the figure. The transcription start site is the nucleotide T which corresponds to an A in the coding strand.

There is no significant sequence homology in the promoter region between the rice root and spinach leaf FNR genes, although the protein sequences are about 50% identical as a whole and much higher homologies are conserved in the cofactor binding regions. The lack of substantial similarity in the 5' flanking regions between the two genes suggests that the transcription regulation might be different between two types of the FNR genes.

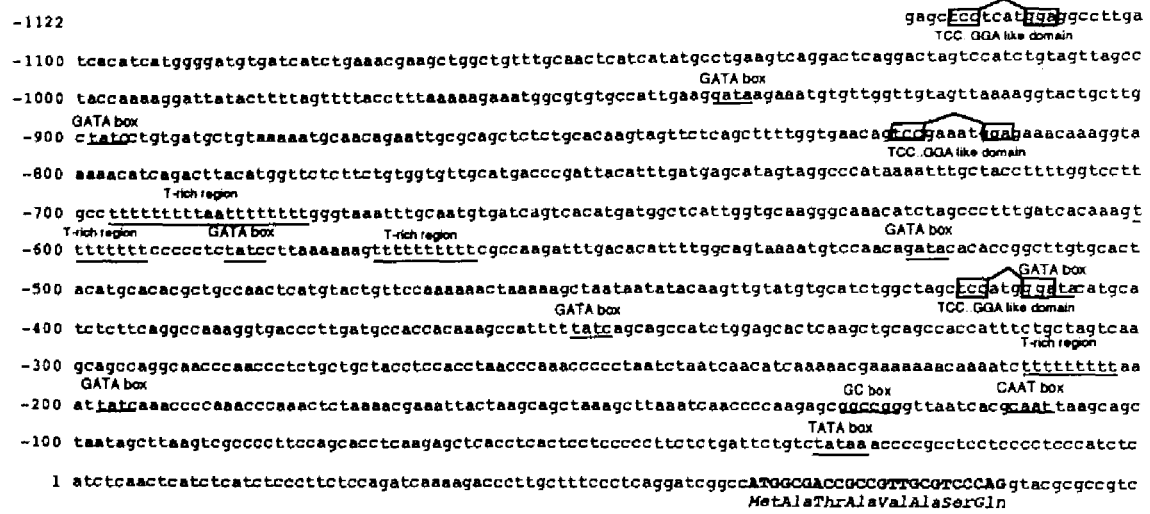
Nucleotide sequence of the 5' upstream region of the rice root FNR gene is shown in Figure 6-4, A. A TATA box (TATAA) and CAAT box (CAAT) are found at -30 and -112 in the 5' flanking region, respectively, as found in many eukaryotic promoters. A Sp 1-binding GC box-like sequence (GGCCGG) is also noticed at -128.

It is interesting to find that seven GATA-boxes are located at -940/-937, -899/-896, -586/-583, -522/-519, -410/-407, -353/-349 and -198/-195 in the root FNR gene (Figure 6-4, A). The GATA motif has been characterized to be the binding site for the transcription factor, NIT2, the gene product of *N. crassa nit-2* (Feng et al. 1993). Detailed studies demonstrated that high affinity NIT-2 binding site of *nit-3* gene (the structural gene of NR in *N. crassa*) contains at least two closely spaced GATA core elements (Chiang and Marzluf 1994a and b). NIT2 mediates global nitrogen repression/derepression in *N. crassa*.

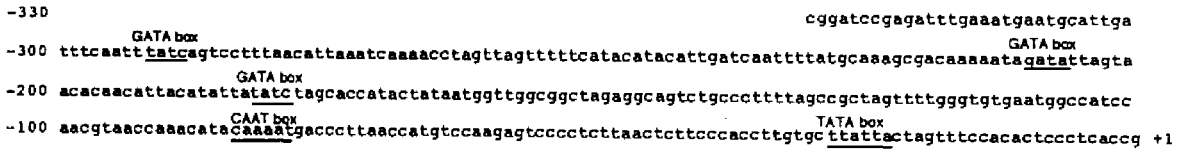
The promoter region of the root FNR gene contains another possible binding sites, TCC..GGA-like sequences at three positions (-1118/-1109, -823/-813, -417/-409) (Figure 6-4, A). TCC..GGA palindromic sequence has been revealed to be the binding site for the NIT4, the gene product of *N. crassa nit-4*, known as the pathway specific transcription factor of the nitrate assimilation pathway in the fungus. But there are 3-5 bp nucleotides between TCC..GGA palindrome sequence in the rice root FNR gene in contrast to the *nit-3* gene of *N. crassa* in which only 2 bp nucleotides are located between them. It is well established that both NIT2 and NIT4 are required to induce the expression of NR and NiR structural genes in *N. crassa.*

Another characteristic sequence, T-rich or A-rich stretches with at least eight T's or A's are present at four positions at 697/-679, -601/-594, -572/-563, -211/-203 in the 5' upstream sequence of the rice root FNR gene (Figure 6-4, A). This T -rich region is also located at -22/-13 in the *nit-3* gene (Okamoto et al. 1991) and at -399/-381 in the *nit-6* gene (structural gene of NiR) in *N. crassa.* (Exley et al. 1993). Although the T or A-rich regions are suggestive of a function in the transcriptional control, their significance is as yet unknown.

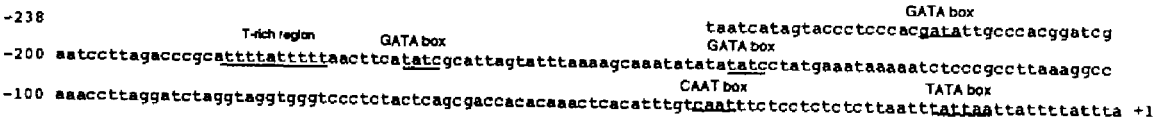
A FNR gene from rice root (-1122/+99)


-1122
TCC GGA like domain
-1100 tcacatcatggggatgtgatcatctgaaacgaagctggctgtttgcaactcatcatatgcctgaagtcaggactcaggactagtccatctgtagttagcc
GATA box
-1000 taccaaaaggattatactttagttttaccttaaaaagaaatggcgtgtgccattgaaggataagaaatgtgttggttgtagttaaaaggtactgcttg
GATA box
-900 ctatcctgtgatgctgtaaaaatgcaacagaattgcgcagctctctgcacaagtagttctcagcttttggtgaacagtccgaaatggaaaacaaaggta
TCC .GGA like domain
-800 aaaacatcagacttacatggttctcttctgtggtgttgcatgacccgattacatttgatgagcatagtaggcccataaaatttgctacctttttggtcctt
T-rich region
-700 gcctttttttttaatttttttttgggtaaatttgcaatgtgatcagtcacatgatggctcattggtgcaagggcaaacatctagccctttgatcacaaagt
T-rich region
GATA box
T-rich region
GATA box
-600 ttttttttccccctctatccttaaaaaagttttttttttcgccaagatttgacacattttggcagtaaaatgtccaacagatacacaccggcttgtgcact
GATA box
-500 acatgcacacgctgccaactcatgtactgttccaaaaaactaaaaagctaataatatacaagttgtatgtgcatctggctagccccgatggtggatacatgca
GATA box
TCC GGA like domain
-400 tctcttcaggccaaaggtgacccttgatgccaccacaaagccatttttatcagcagccatctggagcactcaagctgcagccaccatttctgctagtcaa
T-rich region
-300 gcagccaggcaacccaacccctctgctgctacctccacctaacccaaacccctaatctaatcaacatcaaaaacgaaaaaaacaaaatctttttttttaa
GATA box
GC box
CAAT box
-200 attatcaaaccccaaacccaaactctaaaacgaaattactaagcagctaaagcttaaatcaaccccaagagcgggcgggttaatcacgcaatcaagcagc
TATA box
-100 taatagcttaagtcgcccctccagcacctcaagagctcacctcactcctccccttctctgattctgtctataaaccccgcctcctccccctcccatctc
1 atctcaactcatctcatctccttctccagatcaaaagaccctgctttccctcaggatcggccATGGCGACCGCCGTTGCGTCCCAGgtacgcgccgtc
MetAlaThrAlaValAlaSerGln

B NiR gene from tobacco (-330/+1)



C NR1 gene from Arabidopsis (-238/+1)



D NR2 gene from Arabidopsis (-330/+1)

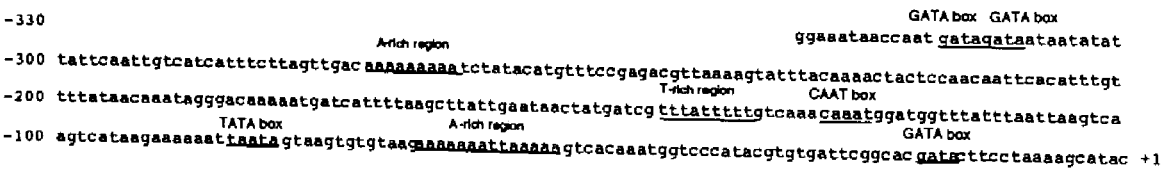


Figure 6-4. Nucleotide sequence of the 5' upstream region of FNR gene from rice root (A), NiR gene from tobacco (B), and NR1 (C) and NR2 (D) gene from *Arabidopsis*. Sequence citation is as follows: A (Aoki and Ida 1994), B (Rastogi et al. 1993), C and D (Lin et al. 1994).

GUS gene expression analysis indicated that a -330/+1 region of the spinach NiR gene promoter directed nitrate-inducible tissue-specific expression in transgenic tobacco (Rastogi et al. 1993). This region contains three GATA-boxes, but no TCC..GGA domain and T-rich region as shown in Figure 6-4, B. Lin et al. (1994) also indicated that a -238/+1 region of the *NR1* gene and -330/+1 region of the *NR2* gene from *Arabidopsis* directed nitrate-induced CAT transcription in transgenic tobacco. The 5' upstream region of *NR1* contains three GATA-boxes and a T-rich region, but no TCC..GGA domain (Figure 6-4, C). The 5' proximal region of *NR2* contains three GATA-boxes, a T-rich region and two A-rich regions, but no TCC..GGA domain (Figure 6-4, D).

It is interesting that several GATA-boxes which are essential for the nitrate induction in *N. crassa* are found in the 5' upstream regions of these nitrate-inducible genes in higher plants (Figure 6-4). The existence of a NIT2-like protein named NTL1 has been reported in *Nicotiana plumbiginifolia* and its full-length cDNA clone was shown to encode a single zinc-finger DNA-binding domain (Daniel-Vedele 1993) as in the case of the NIT2 of *N. crassa* (Marzluf 1993). Thus, it is suggested that NIT2-like transcription factor exists in higher plants and it directs nitrate-inducible transcription of the genes in the nitrate assimilation systems.

Another characteristic sequence, T-rich or A-rich region also exists in the 5' upstream regions of the rice root FNR gene, *nit-3* (Okamoto et al. 1991)and *nit-6* (Exley et al. 1993) in *N. crassa* and *NR1* and *NR2* in *Arabidopsis* (Lin et al. 1994) except for the spinach NiR gene (Rastogi et al. 1993).

Several transcription factors which can bind AT-rich region were indicated recently. Datta and Cashmore (1989) demonstrated that the phosphorylated transcription factor named AT-1 in pea binds to specific AT-rich elements (AT-1 box) within promoters of certain nuclear genes encoding the small subunit of ribulose-1.5-bisphosphate carboxylase and the polypeptide components of the light-harvesting chlorophyll a/b protein complex. A consensus sequence of AATATTTTTATT was derived for the AT-1 box. Jacobsen et al. (1990) demonstrated that three different transcription factors recognized short AT-rich DNA sequences were expressed in different organs of soybean. One factor (NAT 2) was found to be present in mature nodules, another factor (NAT 1) was detected in roots and nodules and the third one (LAT 1) was only observed in leaves. It was indicated that the LAT 1 and the NAT 2 bind the core sequence of 14 bp -TAAATAATAAAATAAA in the promoter region of a nodulin gene, N23 by footprinting analysis. So, it is suggested that a transcription factor which binds to long stretches of AT-rich tracts also exists in higher plants and it directs nitrate-inducible transcription of the genes in nitrate assimilation systems.

Ritchie et al. (1994) indicated that the 3' untranslated region (UTR) of the maize root FNR cDNA was 50% identical to that of the rice root FNR cDNA and the homologous regions

contained the sequence motif, TGTAA. In the 3' UTR of the rice root FNR gene, three TGTAA motifs are localized (Figure 6-2). A TGTAA motif is found in the 3' UTR of the rice embryo FNR cDNA, but no such sequence exists in the 3' UTR of the rice leaf FNR cDNA. The results suggest that the TGTAA motif may be located only in the nonphotosynthetic type FNR cDNA and the motif might play a role in the cell specificity of gene expression and/or nitrate induction.

CHAPTER VII

ANALYSIS OF NUCLEAR PROTEINS WHICH BIND TO THE 5' UPSTREAM REGION OF THE RICE ROOT FERREDOXIN-NADP$^+$ OXIDOREDUCTASE GENE

Little is known about the nitrogen regulatory systems in higher plants, but they have been extensively studied in the fungi, *N. crassa* and *A. nidulans*. Genetic and molecular mechanisms responsible for this regulation have been discussed in higher plants (Crawford and Arst 1993) and fungi (Marzluf 1981; 1993). The *nit-3* gene of *N. crassa* encodes the first enzyme, NR which catalyzes the two electron reduction of nitrate to nitrite in the nitrogen assimilation pathway (Marzluf 1981; Solomonson and Barber 1990). The expression of *nit-3* is highly regulated at the level of mRNA content by metabolic signals, nitrogen derepression, nitrate induction, the positive acting NIT2 and NIT4 transcription factors (Blakely and Srb 1962; Fu and Marzluf 1987b; Okamoto et al. 1991) and the negative acting NMR protein (Sorger et al. 1989). Thus, the *nit-3* gene provides an excellent opportunity to examine the relationship between transcriptional induction and hypersensitive sites in *N. crassa* (Brito et al. 1993).

The nucleotide sequence of *nit-2* gene was translated to yield a protein containing 1,036 amino acid residues with a M_r of approximately 110 kDa and NIT2 contained a single Cys-X_2-Cys-X_{17}-Cys-X_2-Cys type zinc finger DNA binding motif (Fu and Marzluf 1987a; 1990a). Deletion analysis demonstrated that approximately 21% of the NIT2 protein at its C-terminus could be removed without loss of function (Fu and Marzluf 1990a) and site-directed mutagenesis analysis demonstrated that both the single zinc finger motif and the downstream basic region of NIT2 protein were critical for its transactivating function *in vivo* (Fu and Marzluf 1990c).

The *nit-4* gene was isolated and demonstrated to be expressed constitutively to yield a very low abundance 3.5 kb transcript and translated to give a protein of 1090 amino acids. NIT4 protein consisted of $Zn(II)_2Cys_6$ type zinc cluster motif, near its amino acid terminus (Yuan et al. 1991). NIT4 protein also possessed a Gln-rich region and a poly Gln region, both of which were near its C-terminus. A NIT4 protein deleted for the poly Gln region was still functional *in vivo*. However, *nit-4* function was abolished when both the poly Gln region and the Gln-rich domain were deleted. These results suggested that the Gln-rich domain might function in transcriptional activation (Fu et al. 1989; Yuan et al. 1991).

It appeared that NIT2 and NIT4 jointly strongly activated *nit-3* expression, whereas neither factor alone promoted any detectable *nit-3* expression (Fu and Marzluf 1987a). The physiological significance of NIT2 and NIT4 binding elements of *nit-3* gene were estimated by analyzing *nit-3* mRNA expression of 5' promoter deletion clones and mutation of individual NIT2 and NIT4 sites. The results indicated that more than 1 kb upstream of the translational start site including two NIT2 and two NIT4 binding sites were required for *nit-3* mRNA expression (Chiang and Marzluf 1995).

Another important putative nitrogen regulatory gene, *nmr*, appears to act in negative fashion to repress synthesis of NR and the various other nitrogen metabolic enzymes. In *nmr* mutant strains, nitrogen catabolic genes, e.g., NR was expressed constitutively, even in the presence of high concentration of primary nitrogen sources to fully repress synthesis of these enzymes (Tomsett et al. 1981; Dunn-Coleman et al. 1981; Premakumar et al. 1980). The *nmr* gene was isolated (Sorger et al. 1989) and appeared to encode a protein of 488 amino acid residues with a M_r of approximately 54.9 kDa (Young et al. 1990). Recently, a direct interaction between the NIT2 and NMR proteins was demonstrated by the use of two different experimental approaches, suggesting that NMR carried out its negative regulatory role by directory binding to NIT2 and thereby blocking the function of NIT2 by inhibiting its DNA binding activity (Xiao et al. 1995).

It was demonstrated that the FNR mRNA from rice roots was induced by nitrate as described in Chapter V and the 5' upstream region of the root FNR gene contain several GATA-boxes, TCC..GGA domains and T-rich regions as described in Chapter VI. Thus, these findings suggested that the NIT2- and NIT4-like transcription factors exist in higher plants and interact with the 5' proximal regions of the FNR gene. I carried out electrophoreic mobility shift assay (EMSA) for the nuclear extracts of rice. The results indicated that there are some proteins in the nuclear extracts of rice leaf and root, which bind to the 5' upstream regions of the rice root FNR gene containing GATA-box, TCC..GGA domain and T-rich region.

Materials and Methods

Pretreatment of dialysis tubing

Dialysis tubings were pretreated according to Pohl (1990). Dry dialysis tubing (Sankojunyaku) was carefully transferred to 2 l of washing solution (100 mM sodium bicarbonate containing 10 mM EDTA (pH 7.0)). The whole vessel was placed in a shaking

water bath at 60 °C. Gentle agitation was continued for 1 h. The incubation was repeated with fresh solution and this step was repeated three times. The washing solution was replaced with 2 l of ion-exchanged and distilled water and the dialysis tubings were washed for 1 h. This step was repeated three times. Finally, the tubing was transferred to 1 l of ion-exchanged and distilled water including 1 ml chloroform as a preservative and stored at 4 °C.

Preparation of nuclear extracts

Nuclear extracts were prepared from rice roots and leaves according to Koncz et al. (1992). All experiments were done at 4 °C . Total fresh tissues were washed at least three times in 4 l of cold distilled water and linsed in 2 l of nuclei grinding buffer (NGB: 1 M hexylene glycol, 10 mM PIPES-KOH (pH 7.0), 10 mM magnesium chloride, 0.2% Triton X-100, 5 mM 2-ME and 0.8 mM PMSF). The tissues were ground with 2 l of NGB in a Waring blender and mixed gently. The mixture was filtered through 500 μm and 50 μm nylon mesh and the mesh was linsed with 500 ml of NGB. The nuclei were sedimented at 2,000 g for 10 min and the pellet was resuspended gently in 80 ml nuclei wash buffer (NWB: 500 mM hexylene glycol, 10 mM PIPES-KOH (pH 7.0), 10 mM magnesium chloride, 0.2% Triton X-100, 5 mM 2-ME and 0.8 mM PMSF). The mixture was centrifuged 3,000 g for 5 min, removed the supernatant and the pellet was resuspended in 20 ml of nuclei lysis buffer (NLB: 110 mM potassium chloride, 15 mM HEPES-KOH (pH 7.5), 5 mM magnesium chloride, 1 mM DTT, 5 μg/ml antipain and 5 μg/ml leupeptin). Two ml of 4 M ammonium sulfate was added to the mixture in several small aliquots with gentle mixing for 30 min and the particulate material was sedimented by centrifugation at 100,000 g for 90 min. The protein precipitate was resuspended in NLB, adjusted to 0.2-0.5 mg/ml and repeated with ammonium sulfate fractionation to 0.25g/ml. The particulate material was sedimented by centrifugation at 10,000 g for 15 min. The pellet was mixed in 0.5 ml of nuclear extract buffer (NEB: 70 mM potassium chloride, 25 HEPES-KOH pH 7.5, 0.1 mM EDTA, 20% glycerol, 1 mM DTT, 5 μg/ml antipain, 5 μg/ml leupeptin) and the mixture was dialyzed 2 h with 4 changes of 500 ml of NEB. The nuclear extract was freezed by liquid nitrogen and stored at -80 °C.

DNA probes for gel retardation assays

The 5' upstream region (-1122/-131) of the rice root FNR gene was divided in 8 fragments and the position of each probe was as follows: probe 1 (-404/-131), probe 2 (-668/-405), probe 3 (-

804/-668), probe 4 (-1122/-805), probe 1A (-248/-131), probe 1B (-404/-223), probe 1C (-285/-223) and probe NIT4 (-830/-805). Probes 1 to 7, 1A, 1B and 1C were amplified by PCR produce with *Taq* DNA polymerase using *Sal* I -*Eco* RI fragment of the GRFNR5 clone (See Chapter VI) as a template. Each amplified DNA fragment was purified from agarose-LM (Nacalai Tesque). Probe NIT4 was made by annealing two oligonucleotide primers for 90 min at 65 °C. Each probe was labeled 3' end with DIG using a DIG oligonucleotide 3' end labeling kit (Boehringer Mannheim).

Gel retardation assays

EMSA was performed according to Buratowski and Chodosh (1996). Electrophoresis gel was a 5% native polyacrylamide gels (37:1 acrylamide/*N, N'*-methylenebis-acrylamide) with 0.5 × TBE buffer (1 × TBE: 89 mM Tris, 89 mM boric acid, 2 mM EDTA (pH 8.0)). The gels were prerun in 0.5 × TBE buffer without recirculation for 60 min at 10V/cm. The nuclear protein (316 μg for roots, 93.6 μg for leaves) and 50 ng of DIG-labeled DNA probes were incubated for 30 min at 25 °C in a buffer containing 12 mM HEPES-KOH (pH 7.9), 3.5 mM magnesium chloride, 50 mM potassium chloride, 15% glycerol and 2 μg of poly(dI-dC) before being loaded onto the gel. The gels were run in 0.5 × TBE buffer. Probes were transferred to nylon membrane (Hybond-N+, Amersham) after electrophoresis and the signals were detected according to Nomura and Inazawa (1994) with a DIG DNA labeling and detection kit (Boehringer mannheim).

Results and Discussion

From 100 g and 242 g of rice leaves and roots, 312 mg and 126.4 mg of nuclear extract were prepared, respectively. The DNA-protein interaction buffer was the same as used for *nit-3*-NIT2 interaction (Fu and Marzluf 1990b). Poly(dI-dC) which is 40 fold against probe (50 ng) were added in the mixture to prevent nonspecific protein binding.

Figure 7-1 shows the 5' franking region of the rice root FNR gene. The 5' upstream region of the rice root FNR gene was divided into 4 segments corresponding to probes 1 to 4. Probe 1 contains two GATA-boxes and a T-rich region. No TATA-box, CAAT-box and GC-box are located in probe 1 in order to prevent from binding to the transcription factors such as TF-II family. Probe 2 contains three GATA-boxes, a TCC..GGA domain and two T-rich

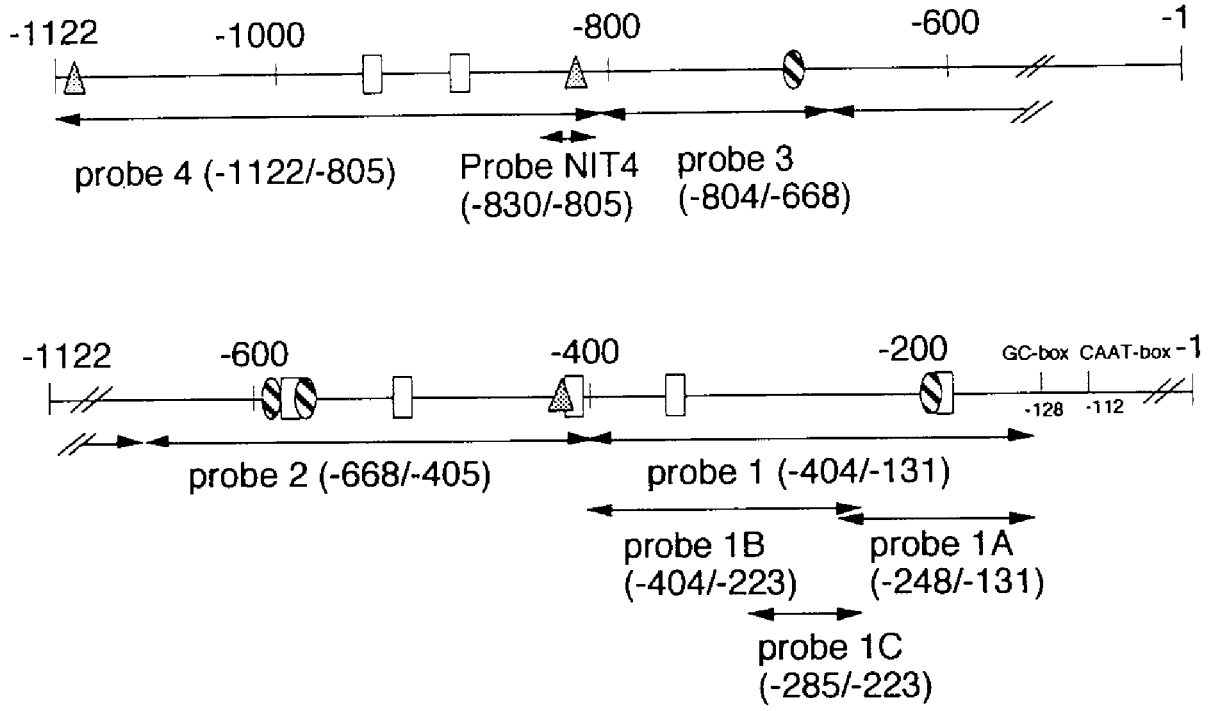
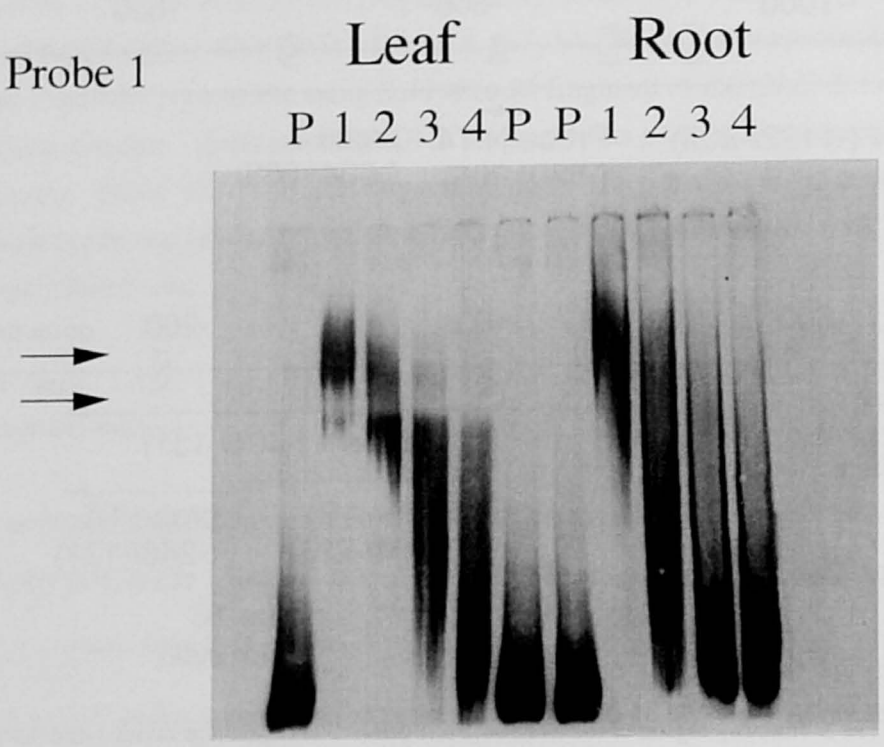


Figure 7-1. The 5' franking region of the rice root FNR gene. The region was divided into 4 segments corresponding to probes1 to 4. Probe 1 was further divided into 1A, 1B and 1C . Probe NIT4 was contained in probe 5. □, GATA box (NIT2binding domain); △ , TCC..GGA domain (NIT4 binding domain and ◊ , T-rich region.

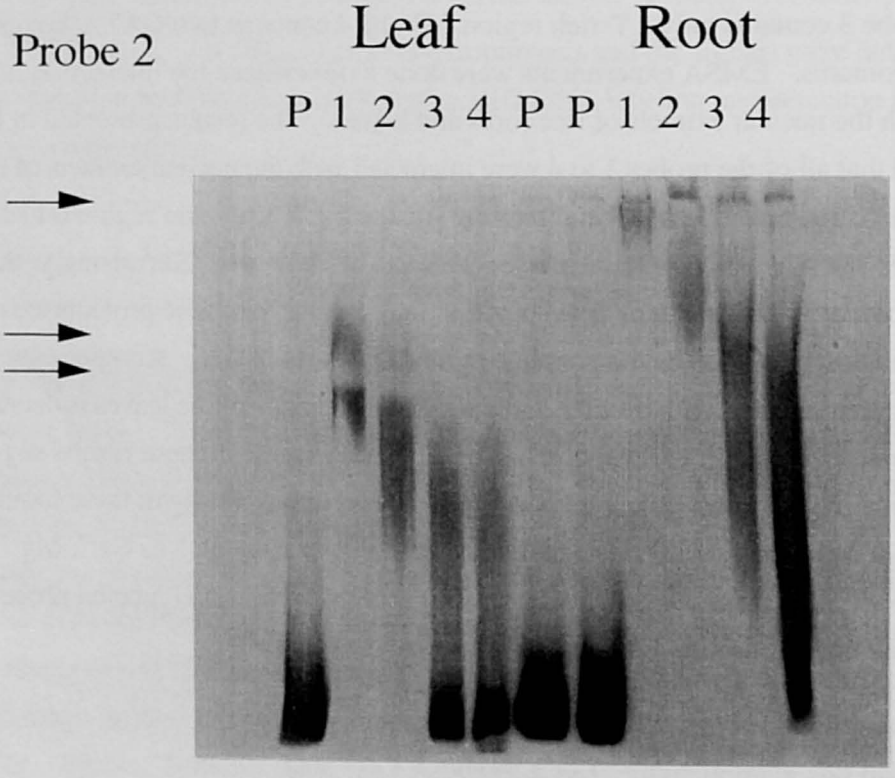
regions. Probe 3 contains only a T-rich region. Probe 4 contains two GATA-boxes and two TCC..GGA domains. EMSA experiments were done to investigate the interaction of these segments with the nuclear extracts of rice roots and leaves. The results presented in Figure 7-2 demonstrated that all of the probes 1 to 4 were interacted with the nuclear extracts of rice roots. The results indicated that at least several binding sites of the 5' upstream region (-1122/-131) are involved in the interaction with the nuclear extracts of rice roots. Surprisingly, the nuclear extracts of rice leaves also contains these proteins, suggesting that these proteins exist in the photosynthetic tissues as well as the nonphotosynthetic tissues of rice. Another experiment indicate that the interaction with probe 1 and the nuclear extracts of rice leaves is decreased when Mg^{2+} was not added in the reaction buffer (data not shown). These results suggest that the nuclear proteins might be expressed constitutively and ubiquitiously in these tissues and nitrate signal or sequential signal (for example intercellular cations such as Ca^{2+}, Mg^{2+} and K^{+} or protein phosphorylation) be able to activate their transcripts. Non-DIG labeled probe competed strongly for the nuclear protein binding of all the probes (Figure 7-2).

A

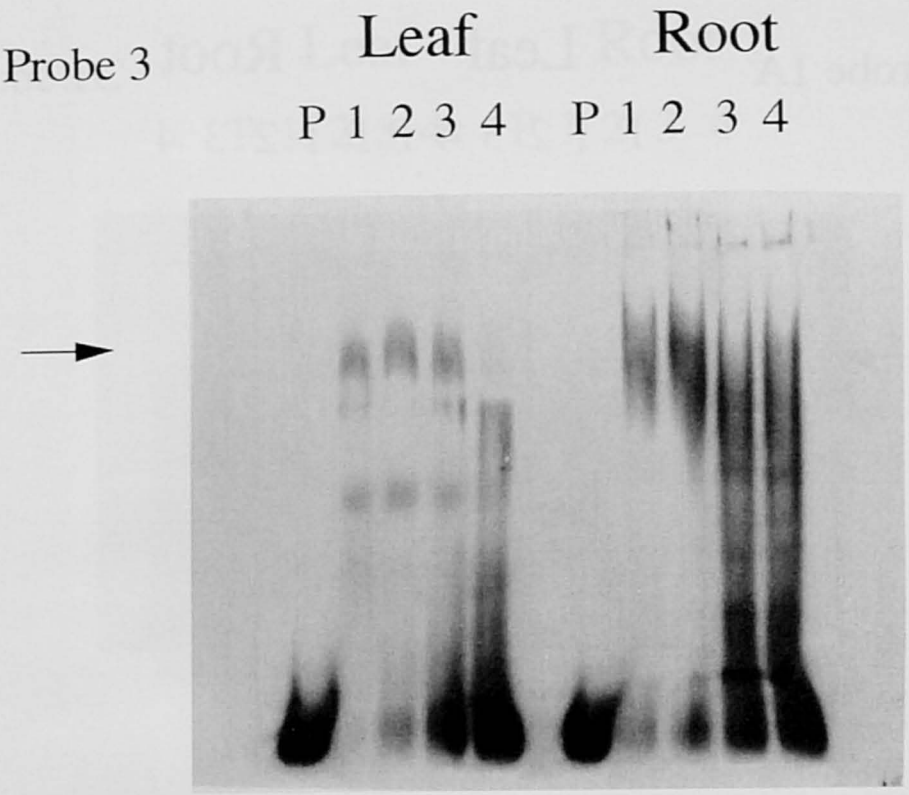
Probe 1



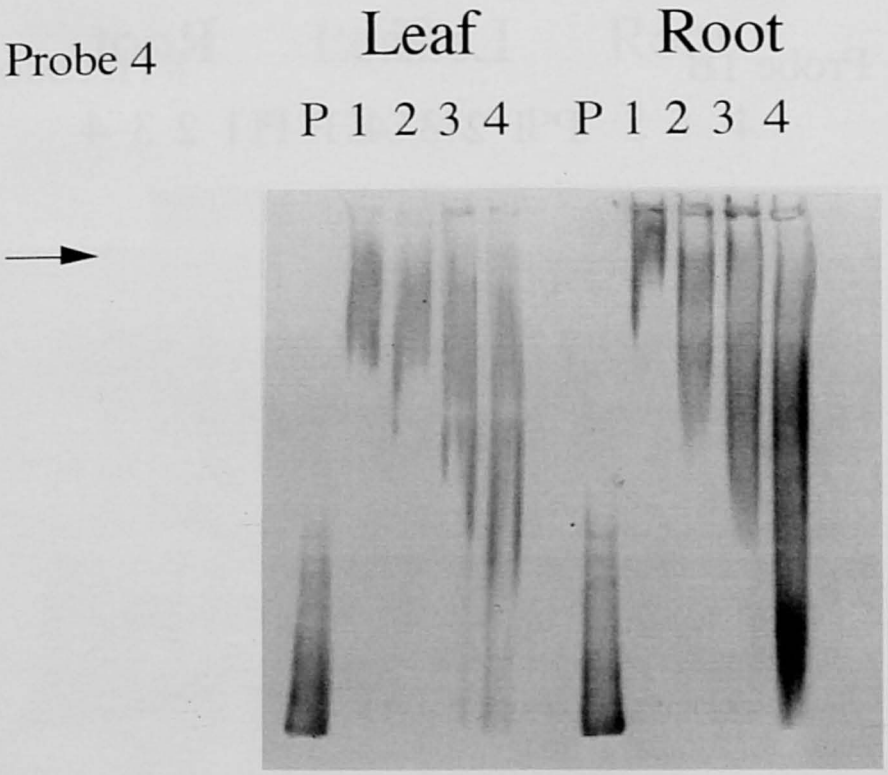
Probe 2



Probe 3



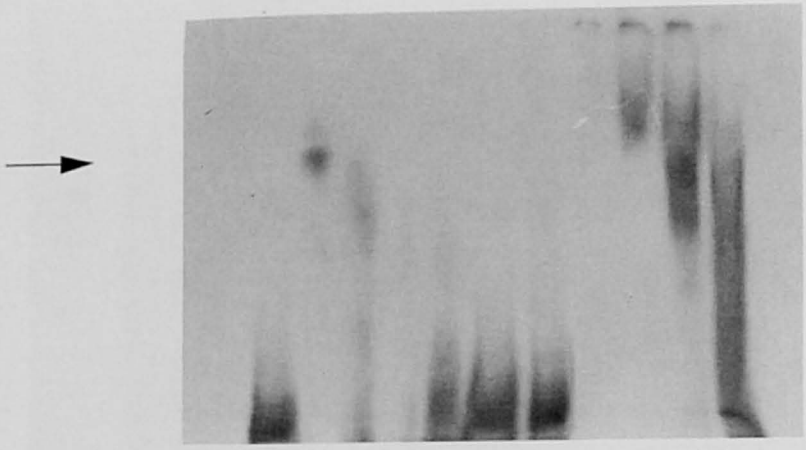
Probe 4



Probe 1A

Leaf Root

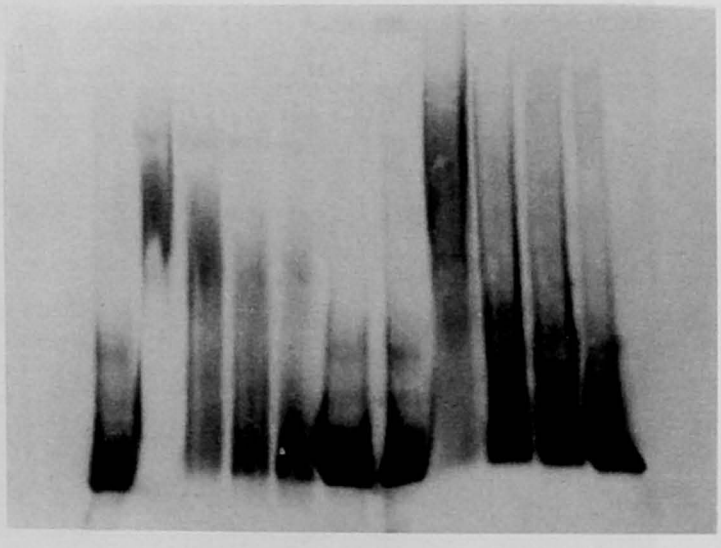
P 1 2 3 4 P P 1 2 3 4



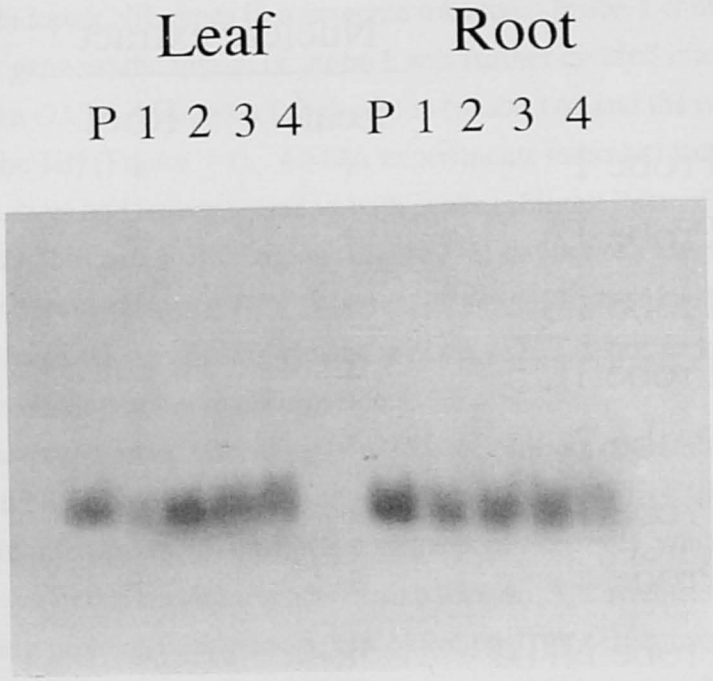
Probe 1B

Leaf Root

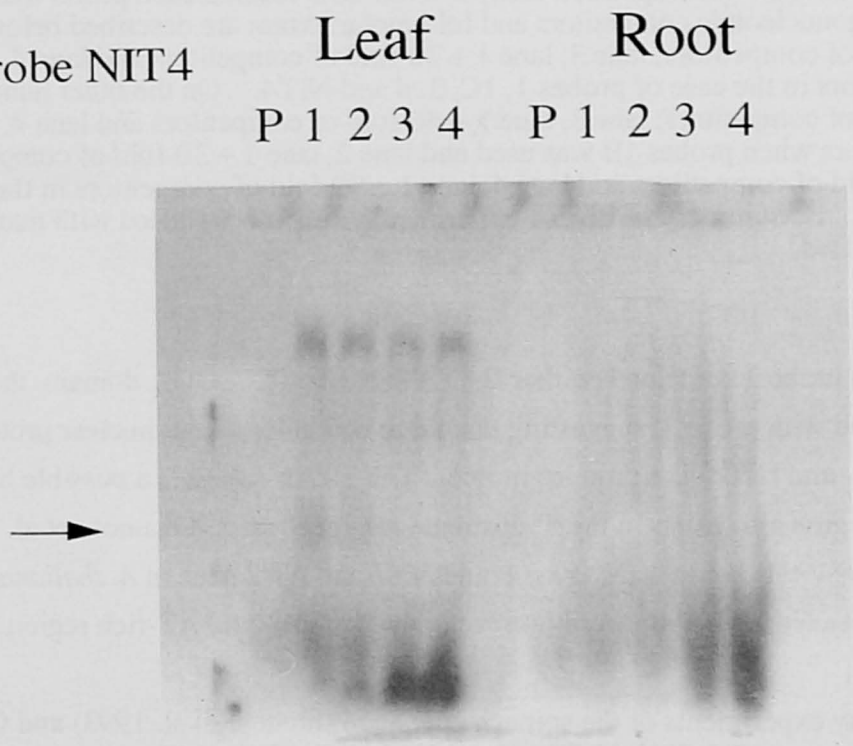
P 1 2 3 4 P P 1 2 3 4



Probe 1C



Probe NIT4



B

	Nuclear extract	
	Leaf	Root
Probe 1	+	+
Probe 1A	+	+
Probe 1B	+	+
Probe 1C	−	−
Probe 2	+	+
Probe 3	+	+
Probe 4	+	+
Probe NIT4	+	−

Figure 7-2. A, Electrophoreic mobility shift assay (EMSA) experiments with 5' upstream DNA fragments of the rice root FNR gene. Length and position of each probe in the 5' upstream region of the gene are described in Figure 7–1. Lane P, free probe (50 ng); lane 1, DIG-labeled probes were incubated with 2 ug of poly(dI-dC) and nuclear extracts from rice roots and leaves and subjected to polyacrylamide gel electrophoresis. An arrow identifies each shifted band. Lanes 2–4, Competition assays. Non-DIG labeled each probe was added as a competitor. Oligonucleotide competitors and fold molar excess are described below. Lane 2, lane 1 + 10-fold of competitors; lane 3, lane 1 + 30-fold of competitors and lane 4, lane 1 + 60-fold of competitors in the case of probes 1, 1C, 3, 4 and NIT4. On the other hand, lane 2, lane 1 + 20-fold of competitors; lane 3, lane 1 + 40-fold of competitors and lane 4, lane 1 + 80-fold of competitors when probes 1B was used and lane 2, lane 1 + 30-fold of competitors; lane 3, lane 1 + 60-fold of competitors and lane 4, lane 1 + 90-fold of competitors in the case of probes 1A and 2. B, Summary of EMSA experiments: +, probes shifted with nuclear extracts; −, probes not shifted.

Although probe 3 contained neither GATA-box nor TCC..GGA domain, the nuclear proteins interacted with probe 3, suggesting that there was still another nuclear proteins in addition to NIT2- and NIT4-like proteins in rice. The T-rich region is a possible binding site, because T-rich region also exists in the 5' upstream region of *nit-3* (Okamoto et al. 1991) and *nit-6* (Exley et al. 1993) genes of *N. crassa* and *NR1* and *NR2* gene in *A. thaliana* (Lin et al. 1994) and it is detected several transcription factors which can bind AT-rich region (See Chapter VI).

GUS assay experiments of the spinach NiR gene (Rastogi et al. 1993) and CAT assay experiments of two *Arabidopsis* NR genes (Lin et al. 1994) indicated that the 5' upstream region between -330 and +1 directed nitrate-inducible tissue specific expression the spinach

NiR gene and two *Arabidopsis* NR genes in transgenic tobacco. Probe 1 contained -330/+1 region of the root FNR gene, so the region of probe 1 was further divided into two segments, one of which contained a GATA-box and a T-rich region (probe 1A) and the other carried a single GATA-box (probe 1B) (Figure 7-1). EMSA experiments indicated that proteins in the nuclear extracts of rice roots and leaves bound to both probes (Figure 7-2). But probe 1C which contains no GATA-box and T-rich region (Figure 7-1) did not react with the nuclear extracts of rice roots and leaves (Figure 7-2). The results suggest that at least two binding sites exist in -404/+131 region of the root FNR gene and probably GATA-box in both probes plays an important role in the binding to the nuclear proteins.

Probe NIT4 was prepared by annealing two 25 base primers containing a TCC..GGA, palindrome sequence in NIT4 binding site (Fu et al. 1995). EMSA analysis for probe NIT4 indicated that no protein interacted with the nuclear extracts of rice roots, whereas the binding of probe NIT4 was observed in the nuclear extracts of rice leaves. The results suggest that there is no TCC..GGA binding proteins in rice roots. Jacobsen et al. (1990) demonstrated that three transcription factors recognized short AT-rich DNA sequences expressed in different organs of soybean (See Chapter VI). It is interesting that transcription factors have tissue-specific expression and control the expression of the enzymes in nitrogen assimilation systems in photosynthetic and nonphotosynthetic tissues.

CHAPTER VIII

CONCLUSION

(1)

Three FNR cDNA clones were isolated from cDNA libraries of rice leaves, roots and embryos and their nucleotide sequences were determined. The FNR cDNA from the monocot leaves was isolated for the first time. The rice leaf FNR cDNA has similar characteristics in the deduced amino acid sequence and M_r to the other leaf FNR cDNAs from higher plants. The rice root FNR cDNA was isolated for the first time from the nonphotosynthetic tissues of higher plants. The rice embryo FNR cDNA is the first report in plant embryos.

The rice FNR genes are expressed in a tissue- or organ-specific manner and these gene products may be related to specific function of each tissue of rice. Expression of the FNR genes may be regulated by different mechanisms such as photoinduction, nitrate induction and developmental stage in each tissue in rice.

(2)

Comparison of the predicted amino acid sequences of three FNR cDNAs revealed that leaf FNR has only 49% identity with the root and embryo counterparts in their mature proteins. On the other hand, root FNR has 90% identity with the embryo enzyme, indicating that they are analogous enzymes as a heterotrophic FNR. Sequence homology of the transit peptide also has this tendency, i.e., the transit peptide of leaf FNR has 28 and 26% identity with that of the root and embryo counterparts, respectively, whereas the transit peptide of root FNR has 57% identity with that of the embryo enzyme.

A phylogenetic tree was constructed, based on amino acid sequence homology of FNRs whose sequences are available at present. Phylogenetic analysis can divide FNRs into 4 groups (group I, FNRs from photosynthetic tissues of higher plants; group II, nonphotosynthetic tissues; group III, green algae and group IV, cyanobacteria) with the exception of *Cyanophora* FNR. Amino acid identities within each group I and group II are more than 80%, whereas sequence homology between group I and group II are less than 50%. Both group I and II are so highly conserved that their sequence homology within the group can not differentiate the monocot and dicot FNRs. Group IV has a low identity to group I as well as to group II, suggesting that cyanobacterial FNR appeared at first in the course of evolution and plant FNRs divided independently into photosynthetic and nonphotosynthetic FNR.

(3)
Genomic Southern hybridization analysis suggested that the leaf, root and embryo FNR gene is a single copy gene, respectively. When the rice leaf FNR cDNA was used as a probe, a single band appeared in 5 different restriction enzymes digests. With the use of the rice root and embryo FNR cDNAs as a probe, 1 to 3 bands appeared in each digest possibly due to cross hybridization, but their hybridization intensity distinguished whether a given band came from the root or embryo FNR gene.

(4)
The root FNR mRNA was induced rapidly after the addition of nitrate in less than 1 h with similar patterns to those known for NR and NiR in higher plant roots, suggesting that root FNR is a member of the nitrate-inducible enzymes in the nitrate assimilation systems in rice roots. The leaf FNR mRNA was induced rapidly by light.

(5)
A genomic clone encoding the rice root FNR was isolated and the nucleotide sequence was determined. Sequence analysis indicated that the root FNR gene consists of 6 exons separated by 5 introns.

Seven GATA-boxes and three TCC..GGA domains are located in the 5' proximal regions of the root FNR gene which are NIT2 and NIT4 binding sites in the NR and NiR genes of *N. crassa* . Another binding site, T-rich region is observed four times in the promoter region of the gene which was present in the *nit-3* and *nit-6* genes encoding NR and NiR in *N. crassa*, respectively.

(6)
Some proteins in the nuclear extracts of rice roots interacted with the promoter region of the root FNR gene containing GATA-box, TCC..GGA domain and T-rich region (probes 1 to 4). These proteins existed also in the nuclear extracts of rice leaves, although the root FNR mRNA was not expressed in the photosynthetic tissues. Probe 1 was further divided into two segments, probes 1A and 1B, both of which contained a GATA-box. EMSA experiments indicated that proteins in the nuclear extracts of rice roots and leaves bound to both probes. The results suggest that GATA-box may play a role in the binding to the nuclear proteins. Probe 1C contained neither GATA-box, TCC..GGA domain nor T-rich region. Probe 1C-protein complex was not observed in the nuclear extracts of rice roots and leaves. The results suggested a possibility of the existence of NIT2- and NIT4-like proteins in rice.

Probe NIT4 is a 25 base DNA fragment which contains a TCC..GGA domain. This probe did not interact with the root nuclear extracts, but with the leaf nuclear extracts, suggesting that NIT4-like protein exists not only in fungus but also in rice leaves.

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